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(71) Applicant (for all designated States except US): MICROBIOLOGICAL RESEARCH AUTHORITY [GB/GB]; CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB).			
(72) Inventors; and			Published
(75) Inventors/Applicants (for US only): SHONE, Clifford, Charles [GB/GB]; Microbiological Research Authority, CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). SUTTON, John, Mark [GB/GB]; Microbiological Research Authority, CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). HALLIS, Bassam [GB/GB]; Microbiological Research Authority, CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). SILMAN, Nigel [GB/GB]; Microbiological Research Authority, CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB).		<i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(74) Agents: SCHLICH, George, William et al.; Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL (GB).			
(54) Title: DELIVERY OF SUPEROXIDE DISMUTASE TO NEURONAL CELLS			
(57) Abstract			
<p>A composition for delivery of superoxide dismutase to neuronal cells comprise a superoxide dismutase linked by a linker to a neuronal cell targeting component, which component comprises a first domain that binds to a neuronal cell and a second domain that translocates the superoxide dismutase into the neuronal cell. After translocation, the linker is cleaved to release superoxide dismutase from the neuronal cell targeting domain. Also described is use of the composition for treatment of oxidative damage to neuronal cells and further targeting of the composition using human mitochondrial leader sequences. A hybrid polypeptide is described that contains a bacterial superoxide dismutase plus a sequence that targets a human mitochondria.</p>			
<pre> graph TD Top[Hybrid Polypeptide] --> Bottom[Protease treatment (e.g. thrombin)] subgraph Top direction TB A[Mn-SOD] --- B[Mitochondrial leader sequence] B --- C[Protease cleavage site (e.g. thrombin)] C --- D[Translocation domain] D --- E[Receptor-binding domain] end subgraph Bottom direction TB A --- F[Translocation domain] F --- G[Receptor-binding domain] end </pre>			

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- 1 -

DELIVERY OF SUPEROXIDE DISMUTASE TO NEURONAL CELLS

The present invention relates to compositions and methods for delivery of superoxide dismutase (SOD) to neuronal cells, and in particular for delivery 5 of SOD to mitochondria in those cells. The present invention also provides methods of making the constructs.

A number of nervous system disorders would benefit considerably from rapid intervention with several therapeutic agents. Examples of such 10 disorders are global, focal or spinal cord ischaemia induced by stroke or injury. Neurones injured by trauma or ischaemia produce elevated levels of transmitter substances which result in high levels of reactive oxygen species. These reactive oxygen species, in high concentrations are toxic to both the neurones and the surrounding cells which potentiates and 15 amplifies the damage process. Rapid therapeutic intervention with agents to reduce oxidative stress in cases of neuronal injury caused by stroke or trauma could therefore significantly limit this secondary damage process. One such potential therapeutic agent is superoxide dismutase which 20 neutralises the harmful effects of the superoxide radicals by converting them to hydrogen peroxide and oxygen.

Francis and co-workers demonstrated that post-ischaemic infusion of Copper/Zinc superoxide dismutase (Cu/Zn-SOD) reduces cerebral infarction following ischaemia/reperfusion in rats (Experimental Neurology (1997) 25 146, 435-443) through the reduction of damaging free-radical oxygen. Lim et al. have shown that administration of Cu/Zn-SOD attenuates the level of reperfusion injury following spinal cord ischaemia in dogs (Ann. Thorac. Surg. (1986) 42, 282-286). Cuevas et al. have similarly demonstrated protective effects of SOD, both on neurological recovery and spinal 30 infarction, in ischaemic reperfusion injury of the rabbit spinal cord (Acta Anat. (1990) 137, 303-310. A major problem in the use of such therapies is the maintenance of useful concentration of the active agent at the site

- 2 -

of trauma. Enzymes such as Cu/Zn-SOD are rapidly cleared from the systemic circulation; in the case of the latter enzyme the $t_{1/2}$ in rat is 4-8 minutes. A number of strategies have been employed to overcome these difficulties. Matsumiaya et al. (Stroke (1991) 22, 1193-1200) conjugated 5 Cu/Zn-SOD to polyethylene glycol to increase its half-life in the blood. Francis and co-workers (Experimental Neurology (1997) 146, 435-443) describe the neuronal delivery of Cu/Zn-SOD by fusion of the enzyme to the binding domain of tetanus toxin. None of these strategies, however, are particularly efficient at delivering the enzyme to the intracellular 10 neuronal compartments where the enzyme can be effective.

In most eukaryotic species, two intracellular forms of superoxide dismutase exist: the Cu/Zn-SOD which is located within the cytoplasmic and nuclear compartments and manganese superoxide dismutase (Mn-SOD) which is 15 located within the mitochondrial matrix. Human Mn-SOD is a tetrameric enzyme and is larger than the dimeric Cu/Zn-SOD. Several studies have shown that decreased Mn-SOD may be associated with one or more chronic diseases such as ovarian cancer (Nishida et al. (1995) Oncology Reports, 2, 643-646) and diabetes (L'Abbe et al., (1994) Proc Soc Exp Biol Med, 207, 206-274). In addition, mice in which the Mn-SOD gene has 20 been knocked out exhibit several novel pathogenic phenotypes including severe anaemia, degeneration of neurones in the basal ganglia and brainstem, and progressive motor disturbances characterised by weakness and rapid fatigue (Lebovitz et al., (1996) Proc Natl Acad Sci USA, 93, 25 9782-9787). In addition these mice showed extensive damage to the neuronal mitochondria. Overexpression of Mn-SOD in cell lines and transgenic mice showed that damage and apoptosis of neurones under oxidative stress was markedly reduced (Keller et al., (1998) Journal of Neuroscience, 18, 687-697). Mitochondrial damage was also reduced. 30 These data showed that superoxide accumulation and subsequent mitochondrial damage play key roles in neuronal death induced by trauma both *in vitro* and *in vivo*. Delivery of agents which reduce the level of

- 3 -

oxidative stress to neuronal cells may therefore reduce neuronal cell death and afford considerable therapeutic benefits.

Mn-SODs of bacterial origin such as that from *Bacillus stearothermophilus* 5 or *B. caldotenax* consist of two subunits and are smaller than the human isoform which is a tetramer. *B. stearothermophilus* and *B. caldotenax* Mn-SOD also have considerably lower immunogenicity than the human isoform which is an advantage for continued therapeutic use. As enzymes for therapeutic applications, however, they suffer from similar drawbacks to 10 other SODs in that very little of the administered enzyme is retained within the tissues where it would be therapeutically beneficial.

The botulinum neurotoxins are a family of seven structurally similar, yet 15 antigenically different, protein toxins whose primary site of action is the neuromuscular junction where they block the release of the transmitter acetylcholine. The action of these toxins on the peripheral nervous system of man and animals results in the syndrome botulism, which is characterised by widespread flaccid muscular paralysis (Shone (1986) in 'Natural Toxicants in Foods', Editor D. Watson, Ellis Harwood, UK). Each 20 of the botulinum neurotoxins consist of two disulphide-linked subunits; a 100 kDa heavy subunit which plays a role in the initial binding and internalisation of the neurotoxin into the nerve ending (Dolly et. al. (1984) Nature, 307, 457-460) and a 50 kDa light subunit which acts intracellularly to block the exocytosis process (McInnes and Dolly (1990) Febs Lett., 261, 25 323-326; de Paiva and Dolly (1990) Febs Lett., 277, 171-174). Thus it is the heavy chains of the botulinum neurotoxins that impart their remarkable neuronal specificity.

Tetanus toxin is structurally very similar to botulinum neurotoxins but its 30 primary site of action is the central nervous system where it blocks the release of inhibitory neurotransmitters from central synapses (Renshaw cells). As described for the botulinum toxins above, it is domains within

- 4 -

the heavy chain of tetanus toxin that bind to receptors on neuronal cells.

The binding and internalisation (translocation) functions of the clostridial neurotoxin (tetanus and botulinum) heavy chains can be assigned to at 5 least two domains within their structures. The initial binding step is energy-independent and appears to be mediated by one or more domains within the H_C fragment of the neurotoxin (C-terminal fragment of approximately 50kDa) (Shone *et al.* (1985), Eur. J. Biochem., 151, 75-82) while the translocation step is energy-dependent and appears to be 10 mediated by one or more domains within the H_N fragment of the neurotoxin (N-terminal fragment of approximately 50kDa).

Isolated heavy chains are non-toxic compared to the native neurotoxins and yet retain the high affinity binding for neuronal cells. Tetanus and the 15 botulinum neurotoxins from most of the seven serotypes, together with their derived heavy chains, have been shown to bind a wide variety of neuronal cell types with high affinities in the nM range (e.g botulinum type B neurotoxin; Evans *et al.* (1986) Eur. J. Biochem. 154, 409-416).

20 Another key characteristic of the binding of these neurotoxins is that tetanus, botulinum A, B, C₁, D, E and F neurotoxins all appear to recognise distinct receptor populations, and collectively the clostridial neurotoxin heavy chains provide high affinity binding ligands that recognise a whole family of receptors that are specific to neuronal cells.

25 However, whilst it is known to provide a fusion of a SOD with a neurotoxin heavy chain, this fusion has been found to be ineffective for delivery of SOD to neuronal cells and inactive in *in vitro* assays for potential therapeutic activity.

30 It is an object of the invention to provide compositions and methods for delivery of SOD to neuronal cells. A further object is to provide

- 5 -

compositions and methods for treatment or at least amelioration of conditions such as ischemic stroke.

Accordingly, the present invention provides a construct for delivering 5 superoxide dismutase (SOD) to neuronal cells. In one aspect of the invention the construct consists of a SOD which has been combined with various functional protein domains to effect efficient targeting to the mitochondria within neuronal cells. The construct of specific embodiments 10 of the invention, described in further detail below, contains the following elements:-

- a SOD which contains a leader sequence for targeting SOD to the mitochondria;
- a dimeric SOD which has low immunogenicity and high stability;
- a SOD which is linked to a domain that effects translocation across 15 lipid membranes;
- a linkage between the SOD and the translocation domain that is cleaved within the neuronal cytosol; and
- a domain which selectively targets the construct to neuronal cells.

20 A first aspect of the invention thus provides a composition for delivery of superoxide dismutase (SOD) to neuronal cells, comprising:-
SOD; linked by a cleavable linker to
a neuronal cell targeting component, comprising a first domain that
binds to a neuronal cell and a second domain that translocates the
25 SOD of the composition into the neuronal cell.

30 The linker is cleavable and thus, in use, after translocation of the SOD into the cell, the linker is cleaved to release SOD from the neuronal cell targeting domain. A suitable linker is a disulphide bridge between cysteine residues, one residue on the SOD and one residue on the neuronal cell targeting component, for example on the second domain. Another example of a linker is a site for a protease found in neuronal cells. In this way, the

- 6 -

linker is such that it is cleaved within the cell, separating the SOD from the other translocated portions of the composition.

By SOD is meant a sequence that has superoxide dismutase activity, and

5 may also comprise a further sequence or sequences conferring additional properties on that portion of the constructs. For example, the SOD optionally also includes a sequence targeting the SOD to mitochondria in a neuronal cell.

10 The SOD may be a hybrid of Mn-SOD and a sequence targeting the hybrid to mitochondria. The SOD may be of bacterial or human origin, or a derivative thereof, and may be comprised of sequences from more than one origin, provided that it has superoxide dismutase activity.

15 The first domain may suitably be selected from (a) neuronal cell binding domains of clostridial toxins; and (b) fragments, variants and derivatives of the domains in (a) that substantially retain the neuronal cell binding activity of the domains of (a). The second domain is suitably selected from (a) domains of clostridial neurotoxins that translocate polypeptide sequences

20 into cells, and (b) fragments, variants and derivatives of the domains of (a) that substantially retain the translocating activity of the domains of (a).

In an embodiment of the invention a construct comprises SOD linked by a disulphide bridge to a neuronal cell targetting component comprising a first domain that binds to a neuronal cell and a second domain that translocates the SOD into the neuronal cell. This construct is made recombinantly as a single polypeptide having a cysteine residue on the SOD which forms a disulphide bridge with a cysteine residue on the second domain. The SOD is covalently linked, initially, to the second domain. Following expression of this single polypeptide SOD is cleaved from the second domain leaving the SOD linked only by the disulphide bridge to the rest of the construct.

A second aspect of the invention provides a pharmaceutical composition for treatment of oxidative damage to neuronal cells comprising a composition according to the invention with a pharmaceutically acceptable carrier. This composition may be used to deliver SOD to a neuronal cell, 5 for example by administration of the composition by injection.

A third aspect of the invention provides a method of preparing a composition according to the invention, comprising chemically linking SOD, 10 linker and neuronal cell targeting components. The SOD preferably is free of cysteine residues and the method preferably comprises treating the SOD with a cross-linker which will form a disulphide bridge with a cysteine residue on the neuronal cell targeting domain.

In a further embodiment of the third aspect of the invention, there is 15 provided a method of making a composition according to the invention comprising expressing a DNA that codes for a polypeptide having SOD, a linker, a neuronal cell targeting component. The polypeptide may further comprise a purification sequence and the method may further comprise purifying the polypeptide using this sequence and then cleaving the 20 polypeptide to remove the purification sequence to leave SOD, the linker and the neuronal cell targeting component.

By virtue of the combination of properties defined above, constructs of the 25 invention are surprisingly efficient at transporting SOD to the mitochondria within neuronal cells. The ability of the superoxide dismutase to be translocated into the cytosol by virtue of the 'translocation domain' within the construct and the cleavage of the enzyme from the latter domain within the cell is key to this targeting efficiency. As such the construct of the invention has considerable therapeutic value in treating neuronal diseases 30 which results from oxidative stress and has several advantages over previously described SOD formulations. Mitochondria within cells containing high levels of superoxide radicals are particularly sensitive to

- 8 -

damage and the ability of the construct of the invention to target the SOD to these organelles offers considerable advantage in that the enzyme can act to remove the superoxide radicals where it is most required.

5 The construct of the invention may be used clinically in a variety of neuronal diseases which are caused or augmented by oxidative stress. Such conditions include ischaemic stroke, Parkinson's disease, Huntington's disease and motor neurone diseases. In the case of ischaemia/reperfusion

10 injury caused by stroke or trauma, delivery of the construct of the invention to neurones of the hippocampus may afford considerable therapeutic benefits by reducing neuronal damage and death. Other neuronal diseases where the underlying cause is oxidative stress would also benefit from the therapeutic effects of the construct of the invention.

15 In a preferred aspect of the invention, the SOD is a dimeric, manganese superoxide dismutase (Mn-SOD) which is of bacterial origin and has low immunogenicity and high stability.

20 The use of a bacterial Mn-SOD in constructs has a number of advantages compared to the use of the human Mn-SOD isoform:-

25 . - the low immunogenicity of the bacterial Mn-SOD is advantageous where repeated administration of the construct is required, in which cases the induction of adverse host immune responses is reduced; and

30 . - the smaller size of constructs based on the dimeric bacterial Mn-SOD compared to human Mn-SOD (which is a tetramer) both reduces the likelihood of adverse immune responses and increases the rate of diffusion of the construct to its target tissue.

In exercise of an example of the invention, a bacterial Mn-SOD of low

- 9 -

immunogenicity is derived from either *B. stearothermophilus* (sequence as reported by Brock and Walker (1980) Biochemistry, 19, 2873-2882) or *B. caldotenax* (gene and amino acid sequence as defined by Chambers *et al.*, (1992) FEMS Microbiology Letters, 91, 277-284) to which a mitochondrial leader sequence has been fused to the N-terminus of the protein by recombinant technology. This Mn-SOD-leader hybrid is linked by a disulphide bridge to a translocation domain derived from a bacterial protein toxin, such as botulinum neurotoxin. The translocation domain, in turn is fused to a receptor binding domain derived from a clostridial neurotoxin (botulinum or tetanus). The construct is produced initially as a single polypeptide by recombinant technology and subsequently converted to the construct of the invention by selective cleavage with a proteolytic enzyme. To produce the construct of the invention, a loop motif containing a unique protease site (e.g amino acid sequences specifically cleaved by proteases such as factor Xa, enterokinase, thrombin) and a cysteine residue is introduced between the C-terminus of the Mn-SOD and the N-terminus of the translocation domain such that a disulphide bridge is formed between the Mn-SOD and the translocation domain. Subsequent cleavage of the protease site generates the active construct. The final construct, when analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis in the presence of a reducing agent (e.g. dithiothreitol), dissociates into two major bands, one corresponding to the superoxide dismutase enzyme and a second corresponding to a polypeptide which contains the neuronal binding and translocation domains. In the absence of a reducing agent this dissociation does not occur and the complex is observed as a single major band on the gels.

Modification of Mn-SOD from *B. stearothermophilus* by addition of a mitochondrial targeting sequence offers several advantages over the use of human Mn-SOD which contains its own mitochondrial leader sequence. Firstly, the *B. stearothermophilus* Mn-SOD has a high thermal stability and low immunogenicity which allows administration of several doses of the

- 10 -

enzyme without provoking an immune response from the host that would reduces its efficacy. Secondly, the *B. stearothermophilus* Mn-SOD is a small dimeric enzyme unlike the human Mn-SOD which is a tetramer. Recombinant constructs containing the latter enzyme would therefore have 5 to be considerably larger and more complex in their structure.

In an embodiment of the invention, a DNA encoding a construct of the invention is made up by fusion of following DNA fragments commencing at the 5'end of the gene:-

10 an oligonucleotide encoding a modified human mitochondrial leader sequence (amino acid sequence: MLSRAVC GTSRQLAPALG YLGSRQ (SEQ ID NO:10) or MLSRAVSGT SRLAPALG YLGSRQ (SEQ ID NO:11);

15 an oligonucleotide encoding Mn-SOD from *B. stearothermophilus* (coding for the amino acid sequence as defined in Brock and Walker (1980) Biochemistry, 19, 2873-2882);

20 an oligonucleotide encoding a linker peptide which contains the thrombin protease cleavage site and a cysteine residue for disulphide bridge formation (peptide sequence: CGLVPAGSGP);

25 an oligonucleotide encoding a translocation domain derived from a botulinum neurotoxin (e.g. a DNA fragment coding for amino acid residues 449-871 of botulinum type A neurotoxin, or a DNA fragment coding for amino acid residues 441-858 of botulinum type B neurotoxin, or a DNA fragment coding for amino acid residues 440-864 of botulinum type F neurotoxin); and

30 an oligonucleotide encoding the receptor binding domain of a botulinum neurotoxin or tetanus neurotoxin (e.g. a DNA fragment

- 11 -

coding for amino acid residues 872-1296 of botulinum type A neurotoxin, or a DNA fragment coding for amino acid residues 859-1291 of botulinum type B neurotoxin, or a DNA fragment coding for amino acid residues 865-1278 of botulinum type F neurotoxin, or a 5 DNA fragment coding for amino acid residues 880-1315 of tetanus neurotoxin).

10 The above DNA fragments may be obtained and constructed by standard recombinant DNA methods. Expression and purification of the assembled construct may be obtained with a variety of suitable expression hosts, e.g. *Escherichia coli*, *Bacillus subtilis*.

15 The translocation domain and neuronal binding domain of the construct may also be derived from combination of different clostridial neurotoxins. For example, the construct of the invention may contain a translocation domain derived from botulinum type F neurotoxin and a binding domain derived from botulinum type A neurotoxin.

20 A construct of the invention may be produced using protein chemistry techniques. Mn-SOD derived from *B. stearothermophilus* to which a mitochondrial leader sequence has been fused to the N-terminus of the protein by recombinant technology is modified with a heterobifunctional cross-linking reagent such as N-succinimidyl 3-[2-pyridyldithio] propionate (SPDP). The 25 chemically modified enzyme is then combined to a cell targetting domain which contains the binding and translocation functional domains. The latter may be produced by recombinant technology or purified from the neurotoxins of *Clostridium botulinum* or *Clostridium tetani* by established methods. Chemical coupling of the SPDP-treated Mn-SOD may be accomplished using a free cysteine residue on the polypeptide containing 30 the binding and translocation domains to give a construct of the invention.

- 12 -

Constructs of the invention may be introduced into either neuronal or non-neuronal tissue using methods known in the art. By subsequent specific binding to neuronal cell tissue, the targeted construct will exert its therapeutic effects. Alternatively, the construct may be injected near a site 5 requiring therapeutic intervention, e.g. intrathecal or intracranial injection close to a site of trauma or disease.

The construct of the invention may also be administered with other agents which enhance its delivery to its target tissue. An example of such an 10 agent is one which assists the passage of the construct of the invention through the blood-brain barrier to the central nervous system. The construct of the invention may also be administered in formulations with other therapeutic agents or drugs.

15 The dosage required for the construct of the invention will depend upon the application and could vary between 1 μ g/kg to 100mg/kg of body weight. The construct of the invention may be produced as a suspension, emulsion, solution or as a freeze dried powder depending on the application and properties of the release vehicle and its therapeutic contents. The construct 20 of the invention may be resuspended or diluted in a variety of pharmaceutically acceptable liquids depending on the application.

25 "Clostridial neurotoxin" means a neurotoxin corresponding to tetanus neurotoxin or one of the seven botulinum neurotoxin serotypes (type A, B, C₁, D, E, F or G).

30 "Bind" in relation to the clostridial binding fragments, means the interaction between the clostridial fragment and one or more cell surface receptors or markers which results in localisation of the binding fragment or construct in the vicinity of the cell.

"Binding domain" of botulinum or tetanus neurotoxins means a domain of

- 13 -

the toxin which retains the property of being able to bind the receptors on neuronal cells in a similar manner to the intact neurotoxin and encompasses native domains and fragments, variants and derivatives that retain this binding function. This property of the binding domain can be assessed in 5 competitive binding assays. In such assays, radiolabelled neurotoxin (e.g botulinum type A neurotoxin) is contacted with neuronal cells in the presence of various concentrations of non-radiolabelled fragment representing the 'binding domain' of the neurotoxin. The ligand mixture is incubated with the cells, at low temperature (0-3°C) to prevent ligand 10 internalisation, during which competition between the radiolabelled neurotoxin and non-labelled 'binding domain' fragment may occur. In such assays when the unlabelled ligand used is binding domain of botulinum type A neurotoxin (residues 872-1296), the radiolabelled botulinum type A neurotoxin will be displaced from the neuronal cell receptors as the 15 concentration of its non-labelled 'binding domain' is increased. The competition curve obtained in this case will therefore be representative of the behaviour of a 'binding domain' fragment being able to bind the receptors on neuronal cells in a similar manner to the intact neurotoxin. This property of the binding domain may be used to identify other suitable 20 protein domains which have the desired binding properties. Examples of binding domains derived from clostridial neurotoxins are as follows:-

	Botulinum type A neurotoxin	- amino acid residues (872 - 1296)
	Botulinum type B neurotoxin	- amino acid residues (859 - 1291)
	Botulinum type C neurotoxin	- amino acid residues (867 - 1291)
25	Botulinum type D neurotoxin	- amino acid residues (863 - 1276)
	Botulinum type E neurotoxin	- amino acid residues (846 - 1252)
	Botulinum type F neurotoxin	- amino acid residues (865 - 1278)
	Botulinum type G neurotoxin	- amino acid residues (864 - 1297)
	Tetanus neurotoxin	- amino acid residues (880 - 1315)

30

"Translocation domain" means a domain or fragment of a protein which effects transport of itself and/or other proteins and substances across a

- 14 -

membrane or lipid bilayer and encompasses native domains and fragments, variants and derivatives that retain this binding function. The latter membrane may be that of an endosome where translocation will occur during the process of receptor-mediated endocytosis. Translocation 5 domains can frequently be identified by the property of being able to form measurable pores in lipid membranes at low pH (Shone *et al.* (1987) Eur J. Biochem. 167, 175-180). The latter property of translocation domains may thus be used to identify other protein domains which could function as the translocation domain within the construct of the invention. Examples of 10 translocation domains derived from bacterial neurotoxins are as follows:-

	Botulinum type A neurotoxin	- amino acid residues (449 - 871)
	Botulinum type B neurotoxin	- amino acid residues (441 - 858)
	Botulinum type C neurotoxin	- amino acid residues (442 - 866)
	Botulinum type D neurotoxin	- amino acid residues (446 - 862)
15	Botulinum type E neurotoxin	- amino acid residues (423 - 845)
	Botulinum type F neurotoxin	- amino acid residues (440 - 864)
	Botulinum type G neurotoxin	- amino acid residues (442 - 863)
	Tetanus neurotoxin	- amino acid residues (458 - 879)

20 "Translocation" in relation to translocation domain, means the internalisation events which occur after modified clostridial binding fragments bind to the cell surface. These events lead to the transport of substances into the cytosol of neuronal cells.

25 "Unique protease site" means a protease site incorporated into the construct such that the molecule may be proteolysed at pre-determined sites by a selected protease. The specificity of these proteases is such that cleavage to other parts of the construct does not occur. Examples of unique protease sites are the amino acid sequences cleaved by proteases 30 such as: thrombin, factor Xa, enterokinase.

A fourth aspect of the invention provides a composition for delivery of a

- 15 -

therapeutic agent to neuronal cells, comprising:-

the therapeutic agent; linked by a cleavable linker to
a neuronal cell targeting component, comprising a first domain that
binds to a neuronal cell and a second domain that translocates the
therapeutic agent of the composition into the neuronal cell.

Thus, in use, after translocation of the therapeutic agent into the cell, the
linker is cleaved to release the therapeutic agent from the neuronal cell
targeting domain. Other optional and preferred embodiments of the fourth
10 aspect of the invention are as for the first-third aspects of the invention.

A fifth aspect of the invention provides a polypeptide comprising a
bacterial SOD, or derivative thereof, and a sequence for targeting the
polypeptide to a mitochondria, such as a human mitochondria. The
15 polypeptide may be chemically obtained by synthesis or otherwise or may
be a fusion protein, obtained for example by expression of a nucleotide
coding for the polypeptide.

The invention hence also provides, in a sixth aspect, a nucleotide encoding
20 the polypeptide of the fifth aspect and in a seventh aspect a vector
comprising the nucleotide of the sixth aspect. Also provided in an eighth
aspect is a method of making a polypeptide according to the fifth aspect
comprising expressing the nucleotide sequence of the sixth aspect. In a
ninth aspect is provided a cell comprising the nucleotide sequence of the
25 sixth aspect or the vector of the seventh.

There now follows description of specific embodiments of the invention
illustrated by drawings in which:-

30 Fig. 1 shows schematic examples of novel Mn-SODs derived from
B. stearothermophilus and *B. caldotenax*. Two examples of mitochondrial
leader sequences are shown. In one example, a cysteine residue at position

- 16 -

7 has been mutated to a serine residue. This change enables the production of the construct of the invention without the formation of disulphide bridges in undesirable positions;

5 Figure 2 shows schematic examples of novel Mn-SOD fusion proteins showing the use of peptides and proteins to facilitate purification of the enzyme from the production strain. Various protein and peptide tags (such as histidine-6, S-peptide, maltose-binding protein, calmodulin-binding protein) may be fused to the Mn-SOD to allow rapid purification by affinity 10 chromatography methods. Unique protease sites are incorporated between the purification tag and the Mn-SOD to enable removal of the tag after purification. Protein and peptide tags may be removed by treatment of the fusion protein with the relevant specific protease (e.g. factor Xa, thrombin, enterokinase);

15

Fig. 3 shows a recombinant Mn-SOD construct of the invention. From the N-terminus of the protein, the construct consists of the following components:- (1) a mitochondrial leader (targeting) sequence, (2) a Mn-superoxide dismutase, (3) a loop which contains a unique protease site and which allows disulphide bridge formation, (4) a translocation domain, (5) a neuronal targeting domain. The construct is produced as a single polypeptide; subsequent cleavage with a protease specific for the 'unique protease site' contained within the loop region generates the di-chain construct. Purification tags could be added to the constructs as exemplified 20 in Figure 3;

25 Fig. 4 shows the production of a Mn-SOD construct by chemical methods. The method uses a recombinant Mn-SOD, purified as described in Example 1 and coupled to a polypeptide containing the translocation and 30 binding domains as described in Example 4; and

Fig. 5 shows the results of an example to demonstrate the protective

- 17 -

effects of a construct of the invention on NG108 cells subjected to oxidative stress by the addition of 50 μ M duroquinone for four hours.

The application is also accompanied by a sequence listing in which:-

5

SEQ ID NO: 1 shows the amino acid sequence of Mn-SOD from *B. caldotenax*;

10

SEQ ID NO: 2 shows the amino acid sequence of Mn-SOD from *B. stearothermophilus*;

15

SEQ ID NO: 3 shows the amino acid sequence of a construct of the invention comprising Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype A;

20

SEQ ID NO: 4 shows the amino acid sequence of a construct of the invention comprising Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype B;

25

SEQ ID NO: 5 shows the amino acid sequence of a construct of the invention comprising Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype F;

30

SEQ ID NO: 6 shows the amino acid sequence of a construct of the invention comprising a mitochondrial leader sequence from human Mn-SOD, Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype A;

- 18 -

SEQ ID NO: 7 shows the amino acid sequence of a construct of the invention comprising a mitochondrial leader sequence from human Mn-SOD, Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype B;

5

10

15

20

SEQ ID NO: 8 shows the amino acid sequence of a construct of the invention comprising a mitochondrial leader sequence from human Mn-SOD, Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype F; and

SEQ ID NO: 9 shows the amino acid sequence for a polypeptide comprising a mitochondrial leader sequence from human Mn-SOD and Mn-SOD from *B. stearothermophilus*;

SEQ ID NO: 10 shows the amino acid sequence of a modified human mitochondrial leader sequence; and

SEQ ID NO: 11 shows an amino acid sequence of a modified human mitochondrial leader sequence.

Example 1.

25 **Production and purification of novel *B. stearothermophilus* Mn-SOD containing a mitochondrial leader sequence.**

30 Standard molecular biology protocols were used for all genetic manipulations (eg. Sambrook *et al.* 1989, Molecular Cloning a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). A synthetic gene encoding the mitochondrial targeting sequence (amino acids 1-27) of the human Mn-SOD gene was cloned as

- 19 -

an *Nde*I - *Bam* HI fragment into an expression vector so that the transcriptional start corresponds to the ATG codon within the *Nde*I site. The Mn-SOD gene from *B. stearothermophilus* or *B. caldotenax* was amplified using PCR to give a *Bgl*II site corresponding to the leucine amino acid at position 5 at the 5' end, and a *Bam*HI site outside the stop codon at the 3' end. This *Bs*III-*Bam*HI fragment was cloned into the expression vector carrying the mitochondrial targeting sequence (digested *Bam*HI) to generate "in-frame" gene fusions. In addition to the wild type mitochondrial targeting sequence, a variant was constructed in which the cysteine at position 7 was changed to serine

The recombinant Mn-SOD expressed in pET28a were produced with amino-terminal histidine (6 His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Ni²⁺ charged column or an anti-T7 immunoaffinity column (Smith *et al.* 1988, *Journal of Biological Chemistry*, 263: 7211-7215). Incorporation of a factor Xa protease cleavage between the peptide tag and the expressed Mn-SOD allowed this to be removed after purification. Briefly, cultures of *E.coli* BL21 (DE3) pET28a-Mn-SOD were grown in Terrific broth-kanamycin (30 µgml⁻¹) to an OD₆₀₀ nm of 2.0, and protein expression was induced by the addition of 500µM IPTG for approximately 2 h. Cells were lysed by freeze/thaw followed by sonication, lysates cleared by centrifugation and supernatants loaded onto an anion exchange column (MonoQ™ column on a Fast Protein Liquid Chromatography system; Pharmacia Biotech, Uppsala, Sweden). Eluted recombinant Mn-SOD was then desalted and further purified by affinity chromatography on a chelating sepharose column charged with Ni²⁺ (Pharmacia Biotech, Uppsala, Sweden). After loading proteins onto the column and subsequent washing, the purified Mn-SOD was eluted with imidazole. All buffers used were as specified by the manufacturer.

30

A 'maltose binding protein' purification tag was also employed for the purification of some batches of Mn-SOD. The use of this system is

- 20 -

described in detail in New England Biolabs Instruction Manual "Protein Fusion and Purification System" (ver 3.02).

5 Other tags and protease cleavage site may also be incorporated into the sequence to facilitate purification of Mn-SOD as exemplified in Figure 2.

10 For purification of a novel *B. stearothermophilus* Mn-SOD which was not conjugated to protein purification tag, the following procedure was used. After harvesting, cells were broken by high pressure homogenisation crude extracts were clarified by centrifugation and batch purified on DE-23 cellulose. The fraction eluted with 0.4M NaCl contained the Mn-SOD. This fraction was then further purified by various chromatographic media using 15 the following sequence:-

15 DEAE-Sepharose ion exchange chromatography at pH 8.0; elution of the Mn-SOD with a NaCl gradient;

20 hydroxylapatite chromatography at pH 6.8; elution of Mn-SOD with a phosphate gradient at pH 6.8;

25 ion exchange chromatography on Q-Sepharose at pH 7.5; elution with a NaCl gradient; and

gel filtration on Sephadryl S-200.

25 The purified Mn-SOD may be dialysed against Hepes buffer (0.1M, pH7.4) containing 0.15M NaCl and stored at -80°C.

Example 2.

30 **Preparation and purification of a recombinant Mn-SOD construct of the invention.**

- 21 -

Standard molecular biology protocols were used for all genetic manipulations (eg. Sambrook *et al.* 1989, Molecular Cloning a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Various DNA fragments of the construct were 5 generated using Recursive PCR reactions (Prodromou & Pearl 1992, *Protein Engineering*, 5: 827-829) using self-priming oligonucleotides containing the desired sequence. For the expression of clostridial neurotoxin fragments the codon bias and GC/AT base ratio was adjusted for ease of expression in *E. coli*. Fragments were cloned sequentially into pLitmus 38 (New England 10 Biolabs, Inc., Beverly, MA) to assemble the entire gene. Constructs for expression were sub-cloned into pET28b (Novagen Inc., Madison, WI) replacing the *EcoR1-HindIII* fragment. The ligation reactions were transformed into *E.coli* DH5 α (Life Technologies Inc., Gaithersburg, MD). Plasmid DNA was amplified, purified and screened for the presence of the 15 appropriate sequence (Ausubel *et al.* 1989, Current Protocols in Molecular Biology, John Wiley & Sons, New York). Gene constructions confirmed as possessing the correct sequences were then transformed into the expression host *E. coli* BL21 (DE3) (Studier & Moffatt 1986, *Journal of Molecular Biology*, 189: 113-130).

20 The recombinant constructs expressed in pET28 were produced with amino-terminal histidine (6 His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Ni²⁺ charged column or an anti-T7 immunoaffinity column (Smith *et al.* 1988, *Journal of Biological Chemistry*, 263: 7211-7215). Incorporation of a factor X protease cleavage 25 between the peptide tags and the expressed Mn-SOD Constructs allowed these to be removed after purification. Briefly, cultures of *E.coli* BL21 (DE3) pET28-Mn-SOD Construct were grown in Terrific broth-kanamycin (30 μ gml⁻¹) to an OD₆₀₀ nm of 2.0, and protein expression was induced by 30 the addition of 500 μ M IPTG for approximately 2 h. Cells were lysed by freeze/thaw followed by sonication, lysates cleared by centrifugation and supernatants loaded onto an anion exchange column (MonoQ™ column on

- 22 -

5 a Fast Protein Liquid Chromatography system; Pharmacia Biotech, Uppsala, Sweden). Eluted recombinant Mn-SOD Construct was then desalted and further purified by affinity chromatography on a chelating sepharose column charged with Ni²⁺ (Pharmacia Biotech, Uppsala, Sweden). After loading proteins onto the column and subsequent washing, the purified Construct was eluted with imidazole. All buffers used were as specified by the manufacturer.

10 A 'maltose binding protein' purification tag was also employed for the purification some batches of Mn-SOD Constructs. The use of this system is described in detail in New England Biolabs Instruction Manual "Protein Fusion and Purification System" (ver 3.02).

15 It would also be evident to anyone skilled in the art that other tags and protease cleavage site may also be incorporated into the sequence to facilitate purification as exemplified in Figure 3.

20 The amino sequences of several recombinant Mn-SOD constructs are shown in the sequence listing.

Example 3.

Preparation of botulinum heavy chains by chemical methods.

25 The various serotypes of the clostridial neurotoxins may be prepared and purified from various toxigenic strains of *Clostridium botulinum* and *Clostridium tetani* by methods employing standard protein purification techniques as described previously (Shone and Tranter 1995, Current Topics in Microbiology, 194, 143-160; Springer). Samples of botulinum neurotoxin (1mg/ml) are dialysed against a buffer containing 50mM Tris-HCl pH 8.0, 1M NaCl and 2.5M urea for at least 4 hours at 4°C and then 30 made 100mM with dithiothreitol and incubated for 16h at 22°C. The cloudy

- 23 -

solution which contains precipitated light chain is then centrifuged at 15000 x g for 2 minutes and the supernatant fluid containing the heavy chain retained and dialysed against 50mM Hepes pH 7.5 containing 0.2M NaCl and 5mM dithiothreitol for at least 4 hours at 4°C. The dialysed
5 heavy chain is centrifuged at 15000 x g for 2 minutes and the supernatant retained and dialysed thoroughly against 50mM Hepes pH 7.5 buffer containing 0.2M NaCl and stored at -70°C. The latter procedure yields heavy chain >95% pure with a free cysteine residue which can be used for chemical coupling purposes. Biological (binding) activity of the heavy
10 chain may be assayed as described in Example 5.

The heavy chains of the botulinum neurotoxins may also be produced by chromatography on QAE Sephadex as described by the methods in Shone and Tranter (1995) (Current Topics in Microbiology, 194, 143-160; 15 Springer).

Example 4

Production of Mn-SOD constructs by chemical methods

20 *B. stearothermophilus* Mn-SOD fused to a mitochondrial leader sequence was purified as described in Example 1. The Mn-SOD was chemically modified by treatment with a 3-5 molar excess of N-succinimidyl 3-[2-pyridyldithio] propionate (SPDP) in 0.05M Hepes buffer pH 7.0 containing 0.1M NaCl for 60 min at 22°C. The excess SPDP was removed by dialysis against the same buffer at 4°C for 16h. The substituted SOD was then mixed in a 1:2.5 molar ratio with heavy chain purified from *Clostridium botulinum* type A neurotoxin purified as described in Example 3 and incubated at 4°C for 16h. During the incubation period the Mn-SOD was conjugated to the botulinum heavy chain fragment by free sulphhydryl groups (see Figure 4). After incubation, the Mn-SOD-construct was purified
25 by gel filtration chromatography on Sephadex G200.
30

- 24 -

Constructs of the invention may also be formed by the above method using polypeptides containing the translocation and binding domains that have been produced by recombinant technology as outlined in Example 2.

5 **Example 5.**

**Assay of the biological activity of constructs -
demonstration of high affinity binding to neuronal cells.**

10 Clostridial neurotoxins may be labelled with 125-iodine using chloramine-T and its binding to various cells assessed by standard methods such as described in Evans *et al.* 1986, Eur J. Biochem., 154, 409 or Wadsworth *et al.* 1990, Biochem. J. 268, 123). In these experiments the ability of Mn-SOD constructs to compete with native clostridial neurotoxins for receptors present on neuronal cells or brain synaptosomes was assessed.

15 All binding experiments were carried out in binding buffers. For the botulinum neurotoxins this buffer consisted of: 50mM HEPES pH 7.0, 30mM NaCl, 0.25% sucrose, 0.25% bovine serum albumin. For tetanus toxin, the binding buffer was: 0.05M tris-acetate pH 6.0 containing 0.6% bovine serum albumin. In a typical binding experiment the radiolabelled clostridial neurotoxin was held at a fixed concentration of between 1-20nM. Reaction mixtures were prepared by mixing the radiolabelled toxin with various concentrations of unlabelled neurotoxin or construct. The reaction mixture were then added to neuronal cells or rat brain synaptosomes and then incubated at 0-3°C for 2hr. After this period the neuronal cells or synaptosomes were washed twice with binding ice-cold binding buffer and the amount of labelled clostridial neurotoxin bound to cells or synaptosomes was assessed by γ -counting. In an experiment using an Mn-SOD construct which contained the binding domain from botulinum

20 type A neurotoxin, the construct was found to compete with ^{125}I -labelled botulinum type A neurotoxin for neuronal cell receptors in a similar manner to unlabelled native botulinum type A neurotoxin. These data showed that

25

30

- 25 -

the construct had retained binding properties of the native neurotoxin.

Example 6

5 **Assay of the biological activity of constructs – measurement of the Mn-SOD activity.**

Mn-SOD activity in samples and constructs was measured by a modification (Brehm *et al.* (1991) *Appl. Microbiol. Biotechnol.*, 36, 358-363) 10 of the procedure described by McCord and Fridovich (*J. Biol. Chem.* (1969), 244, 6049-6055). Aliquots (20 μ l) of samples or constructs containing Mn-SOD were added to 1ml of 0.05M potassium phosphate buffer pH 7.5 containing 1 \times 10⁻⁴ M EDTA, 2.5 \times 10⁻⁵ M ferricytochrome C and 7 \times 10⁻³ M sodium xanthine in a thermostatted cuvette at 30°C. 15 Sufficient xanthine oxidase was added to produce a rate of reduction of the ferricytochrome C at 550nm of approx. 0.1 absorbance units/minute in the absence of Mn-SOD. Under these conditions the amount Mn-SOD that was required to reduce the rate of reduction of ferricytochrome C by 50% was defined as one unit of activity.

20 Using such assays the Mn-SOD activity within constructs was assessed.

Example 7

25 **Demonstration of the targeting of Mn-SOD to the mitochondria of neuronal cells by constructs of the invention**

Mn-SOD construct containing the translocation and targeting domains derived from botulinum type A neurotoxin was incubated at various 30 concentrations (0.01-10 μ M final concentration) with a neuroblastoma cell line NG108. Incubations were carried out over a 6h period or overnight at 37°C. In some experiments, construct radiolabelled with ¹²⁵Iodine was

- 26 -

used. After incubation with the construct cells, were removed from culture flasks by gentle scraping and centrifuged at 200 x g. Cells were then resuspended in breaking buffer (0.6M mannitol, 20mM hepes pH 7.4 and 1mM phenylmethylsulphonyl chloride) and homogenised in a Dounce 5 homogeniser. The homogenate was centrifuged at 200 x g for 5min and then the supernatant fluid recovered and centrifuged at 8000 x g for 10min. The 200 x g pellet (nuclear fraction) were pooled and resuspended in phosphate buffered saline. The 8000 x g pellets (mitochondrial fraction) were also pooled and resuspended in phosphate buffered saline. The 10 supernatant fluid was saved and used to represent the cytosolic fraction.

Analysis of the sub-cellular distribution of Mn-SOD was carried out by Western blot analysis and, where radiolabelled construct was used, by analysis of the ^{125}I -labelled construct components by γ -counting and by 15 autoradiography of cell fractions which had been separated by electrophoresis on SDS-polyacrylamide gels. For Western blot analysis, proteins in the cell fractions were separated by electrophoreses on SDS-polyacrylamide gels and then transferred to nitrocellulose membrane as described previously (Towbin *et al.* Proc.(1979) Natl. Acad. Sci. USA, 76, 20 4350). The presence of Mn-SOD in protein bands on nitrocellulose membranes was assessed by incubation with rabbit anti- Mn-SOD antibody followed by washing and incubation with anti-rabbit peroxidase conjugate. 25 Addition of peroxidase substrates (3,3',5,5'- tetramethyl benzidine and H_2O_2) allowed visualisation and quantitation of the Mn-SOD in the various sub-cellular protein fractions. An enhanced chemiluminescence system (Amersham International) was also used in some experiments to increase the sensitivity.

Example 8.

30

Formulation of the Mn-SOD construct for clinical use.

- 27 -

In a formulation of the Mn-SOD construct for clinical use, recombinant Mn-SOD construct would be prepared under current Good Manufacturing Procedures. The construct would be transferred, by dialysis, to a solution to give the product stability during freeze-drying. Such a formulation may 5 contain Mn-SOD construct (10 mg/ml) in 5mM HEPES buffer (pH 7.2), 50mM NaCl, 1% lactose. The solution, after sterile filtration, would be aliquotted, freeze-dried and stored under nitrogen at -20°C.

10 **Example 9.**
Use of an Mn-SOD construct to treat stroke.

15 In a typical case of a middle aged or elderly man diagnosed as suffering from stroke, treatment with an Mn-SOD construct would begin immediately, ideally within 6 hours of the stroke occurring. Doses of the Mn-SOD construct (e.g. 100mg) reconstituted in a sterile saline solution would be administered intravenously. Further doses of the construct would 20 be administered daily for 5-10 days. Such a patient would be expected to display reduced levels of ischaemia/reperfusion damage as assessed by magnetic resonance imaging compared to a similarly affected patient receiving no treatment. Relative improvements to muscle strength and co-ordination (MRC motor score) would be expected to be observed over the subsequent 12 month period.

25 **Example 10.**
Method to Demonstrate Neuroprotection of Cells by MnSOD-Heavy Chain Conjugates

30 Neuroblastoma cell line NG108-15 (*Nature* (1998) 336:p185) were seeded at a density of 3×10^4 cells/ml in 96 well microtitre plates coated with poly-D-lysine. Plates were grown for 3 days at 37°C in a CO₂ incubator (5% CO₂

- 28 -

95% air). MnSOD or leader-MnSOD conjugates were prepared with purified heavy chain of botulinum neurotoxin serotype A (BoNT/A HC) as described. The concentration of SOD was estimated and the conjugate diluted to give the specified amount of conjugate in a total volume of 200 μ l serum free medium. Conjugate was added to wells in the presence or absence of 5 56mM KCl, 2mM CaCl₂. The cells were incubated with conjugate for 1 hour. The conjugate was replaced with either serum free medium or serum free medium containing 50 μ M duroquinone and incubated at 37°C for 4 hours in the CO₂ incubator to induce oxidative stress. The media was 10 removed after 4 hours and replaced with the dye 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.25mg/ml in serum free medium and incubated for 2 hours (according to the method of Mattson, MP, *et al* (1995) *Methods in Cell Biology* 46:187-216) The conversion of MTT to formazan dye crystals has been shown to 15 be related to mitochondrial respiratory chain activity (Musser, DA, and Oseroff, AR (1994) *Journal of Immunology* 59:621-626). MTT was removed and crystals solubilised with dimethylsulfoxide (DMSO). Absorbance at 570nm was measured using a Labsystems Multiskan Plus MkII spectrophotometer and the results shown in Figure 5.

20

Figure 5 shows that a construct of the invention was able to protect neuronal cells against the mitochondrial - focused oxidative stress produced by duroquinone.

25

Example 11.

Preparation and purification of recombinant MnSOD and leader sequence MnSOD

30

Standard molecular biology protocols were used for all genetic manipulations (Sambrook *et al* 1989, Molecular cloning; A laboratory manual. Second Edition, Cold Spring Harbor Laboratory Press, New York.).

- 29 -

The MnSOD gene from either *B. stearothermophilus* or *B.caldotenax* was amplified by PCR to engineer a *Bam*HI site (resulting in the replacement of nucleotides 1-15). A synthetic oligonucleotide corresponding to the mitochondrial leader sequence of human MnSOD (bases 1-81 of the human gene) was subcloned into the *Bam*HI site to generate leader-MnSOD. PCR was used to add a Factor Xa cleavage site immediately adjacent to the methionine at the start of the leader sequence. Similarly a Factor Xa cleavage site was engineered immediately adjacent to the methionine at the start of the native MnSOD gene. Constructs were sequenced to confirm the presence of the correct sequence. Constructs for expression were subcloned into the expression vector pET28a (Novagen Inc, Madison, WI) as an *Eco*RI fragment and the orientation of the fragments checked. Clones with confirmed sequences were used to transform expression host *E.coli* BL21 (DE3) (Studier and Moffatt 1986 *Journal of Molecular Biology* 189:113-130). Examples 2 and 3 above provide detailed methods.

The recombinant proteins expressed from pET28a contain amino-terminal histidine (6-His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Cu²⁺ charged metal chelate column or an anti-T7 immunoaffinity column. Incorporation of the Factor Xa site between the peptide tags and the start of either MnSOD or leader MnSOD allows the precise removal of the peptide tags after purification. Cultures of *E.coli* BL21(DE3) pET28a-MnSOD or BL21(DE3) pET28a-leader-MnSOD were grown in Terrific Broth containing 30µg/ml kanamycin and 0.5% (w/v) glucose to an OD₆₀₀ of 2.0 and protein expression was induced with 500µM IPTG for 2 hours. Cells were lysed by sonication, cell debris pelleted by centrifugation and the supernatant loaded onto a metal chelate column charged with Cu²⁺ (Amersham-Pharmacia Biotech, Uppsala, Sweden). After loading proteins on the column and washing, proteins were eluted using imidazole. All buffers were used as specified by manufacturers. Factor Xa cleavage of the eluted protein was carried out according to manufacturers instructions.

- 30 -

The invention thus provides constructs and methods for delivery of SOD to neuronal cells.

CLAIMS

1. A composition for delivery of superoxide dismutase (SOD) to neuronal cells, comprising:-
 - 5 SOD; linked by a cleavable linker to a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the SOD of the composition into the neuronal cell.
- 10 2. A composition according to Claim 1 for delivery of SOD to mitochondria of neuronal cells wherein the SOD comprises a sequence targeting the SOD to mitochondria in the neuronal cell.
- 15 3. A composition according to Claim 2 wherein the SOD is a hybrid of Mn-SOD and a sequence targeting the hybrid to mitochondria.
4. A composition according to Claim 2 or 3 wherein the mitochondria targeting sequence is derived from human Mn-SOD.
- 20 5. A composition according to any of Claims 1-4 wherein the SOD is bacterial SOD or is derived therefrom.
- 25 6. A composition according to any of Claims 1 to 5 wherein the first domain is selected from (a) neuronal cell binding domains of clostridial toxins; and (b) fragments, variants and derivatives of the domains in (a) that substantially retain the neuronal cell binding activity of the domains of (a).
- 30 7. A composition according to any Claims 1 to 6 wherein the second domain is selected from (a) domains of clostridial neurotoxins that translocate polypeptide sequences into cells, and (b) fragments, variants and derivatives of the domains of (a) that substantially retain the

- 32 -

translocating activity of the domains of (a).

8. A composition according to any of Claims 1 to 7 wherein the linker is a disulphide bridge.

5

9. A pharmaceutical composition for treatment of oxidative damage to neuronal cells comprising a composition according to any of Claims 1 to 8 and a pharmaceutically acceptable carrier.

10 10. A method of delivering SOD to a neuronal cell comprising administering a composition according to Claim 9.

11. A method according to Claim 10 comprising injecting the composition.

15

12. A method of making a composition according to any of Claims 1 to 8 comprising chemically linking SOD, a linker and a neuronal cell targeting component.

20

13. A method of making a composition according to any of Claims 1 to 8 comprising expressing a DNA that codes for a polypeptide having SOD activity, a linker, and a neuronal cell targeting component.

25

14. A method according to claim 13 wherein the polypeptide further comprises a purification sequence and the method further comprises purifying the polypeptide and then cleaving the polypeptide to remove the purification sequence to leave SOD, the linker and the neuronal cell targeting component.

30

15. A composition for delivery of a therapeutic agent to neuronal cells, comprising:-

- 33 -

the therapeutic agent; linked by a cleavable linker to a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the therapeutic agent of the composition into the neuronal cell.

5

16. A polypeptide comprising a bacterial SOD or derivative thereof and a sequence for targeting the polypeptide to a human mitochondria.

10 17. A polypeptide according to Claim 16 wherein the SOD is from *Bacillus*.

18. A polypeptide according to Claim 16 or 17 which is a fusion protein.

19. A nucleotide encoding the polypeptide of any of Claims 16-18.

15

20. A vector comprising the nucleotide of Claim 19.

21. A method of making a polypeptide according to any of Claims 16-18 comprising expressing the nucleotide sequence of Claim 19.

20

22. A cell comprising the nucleotide sequence of Claim 19 or the vector of Claim 20.

1/4

FIG. 1

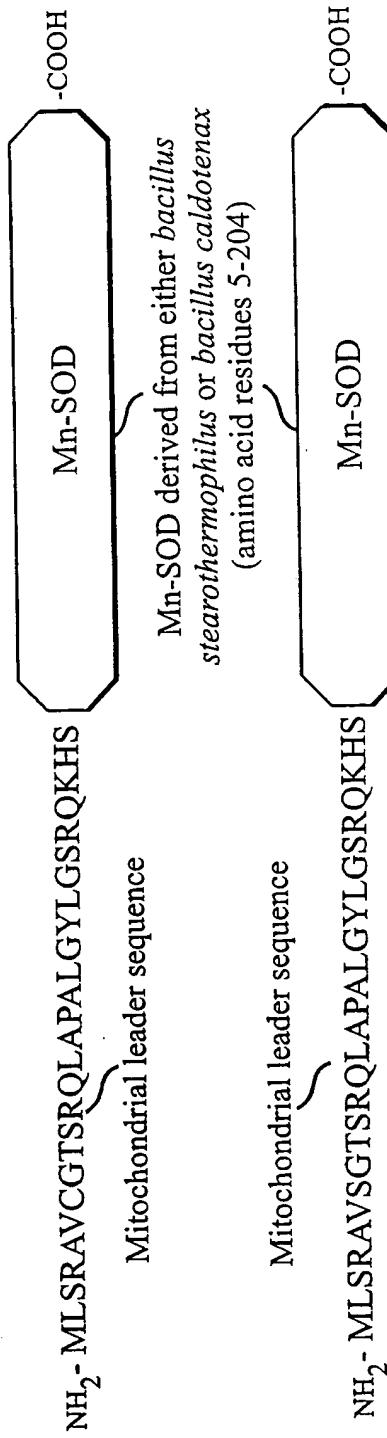
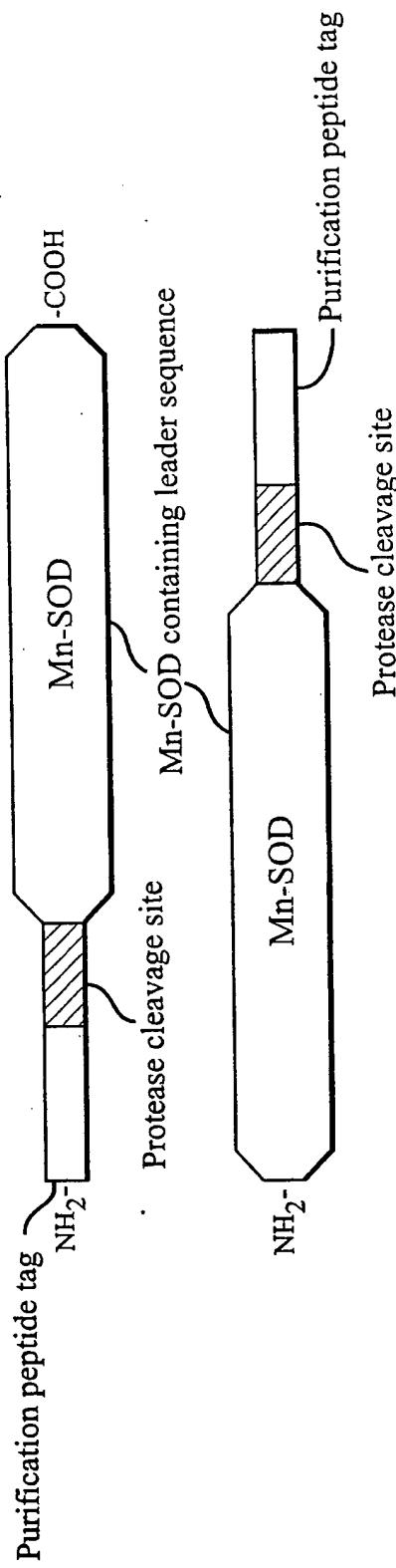
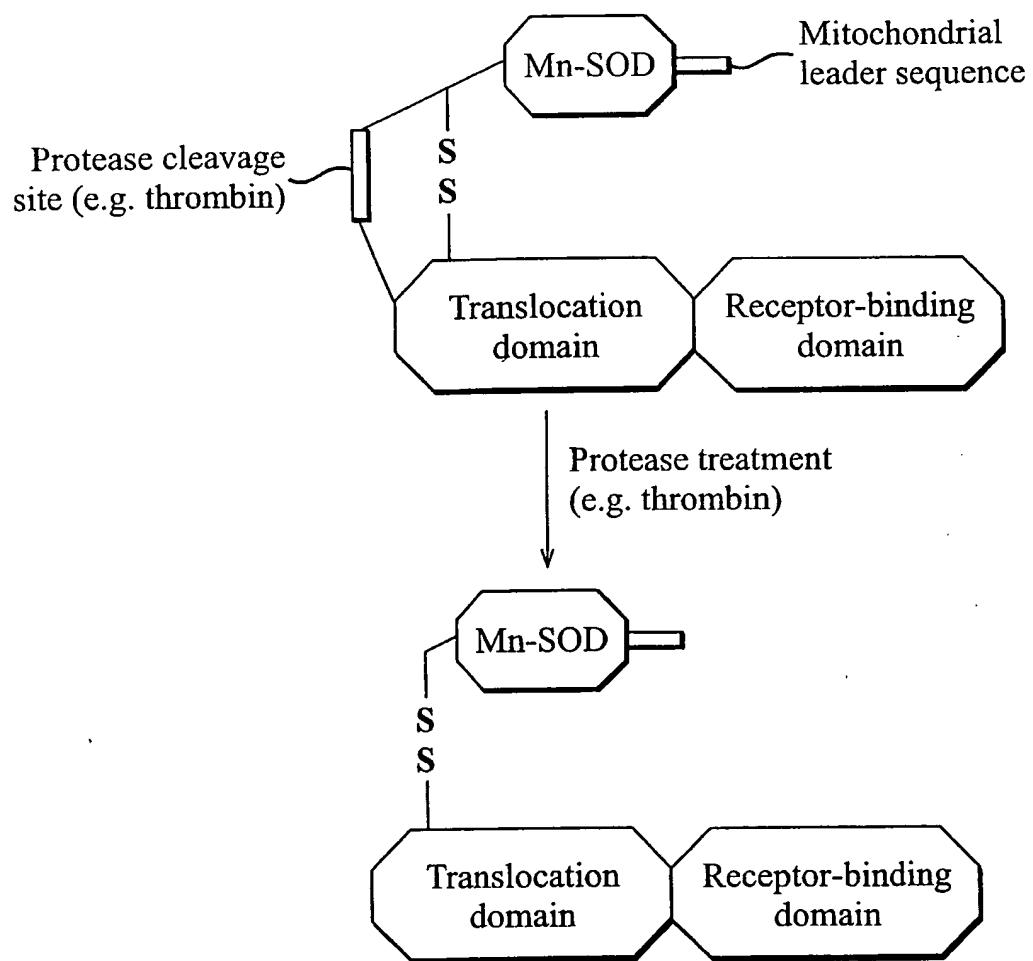


FIG. 2



2/4

FIG. 3



3/4

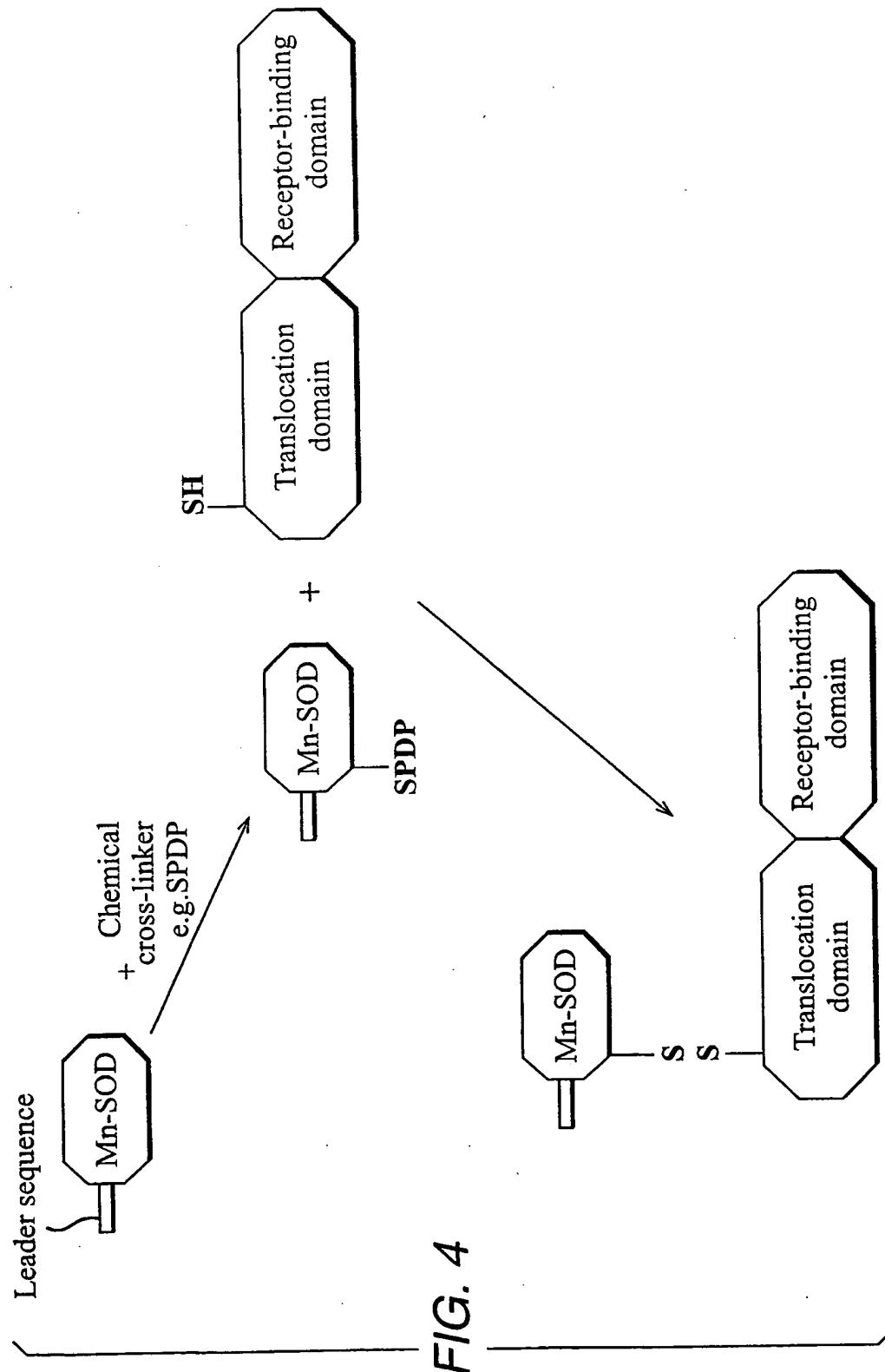
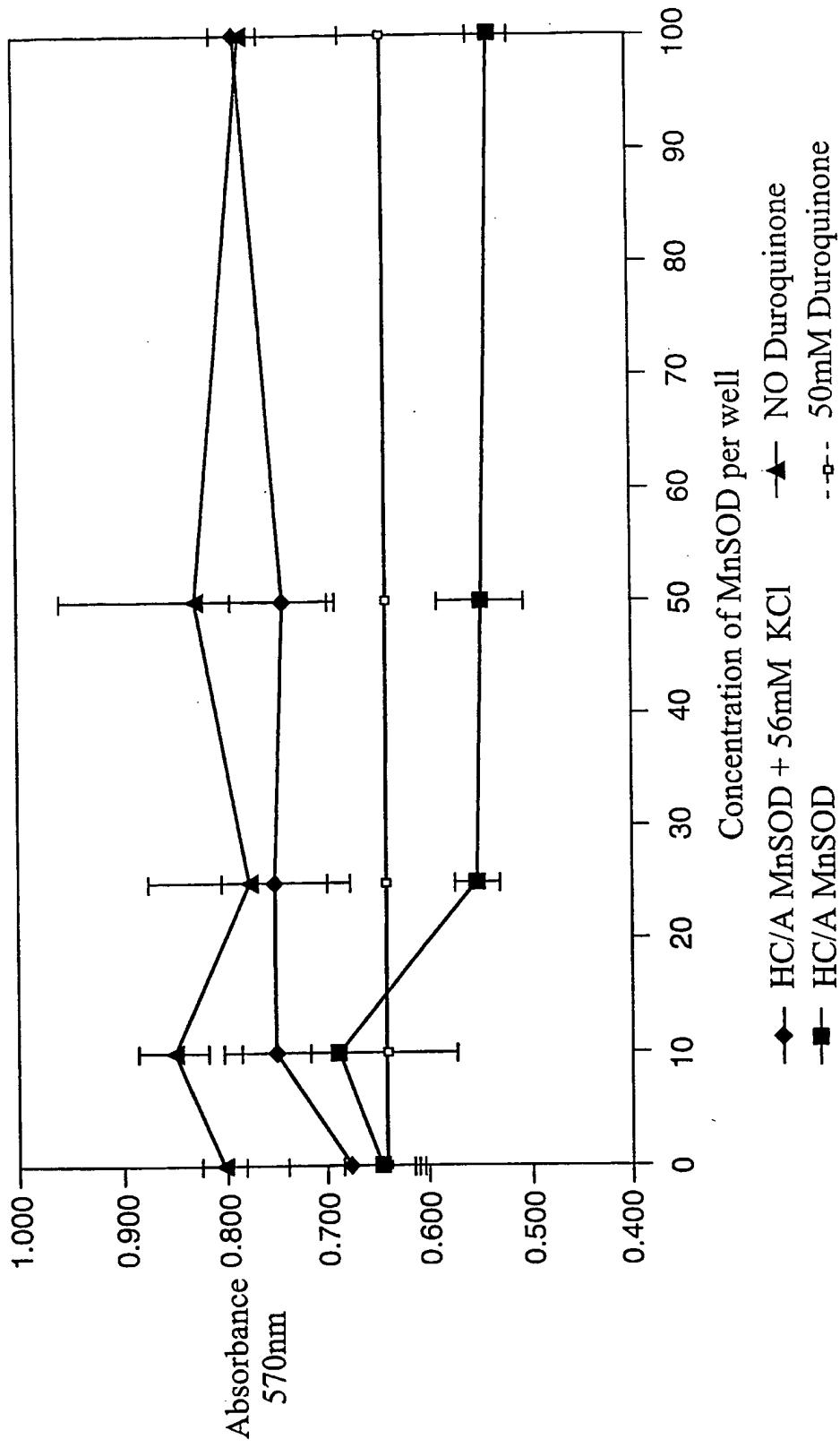


FIG. 4

4/4

FIG. 5 Demonstration of protective effects of HC/A MnSod on NG108 cells subjected to oxidative stress by the addition of 50 μ M duroquinone for 4 hours.



- 1 -

SEQUENCE LISTING

5 <110> Microbiological Research Authority
 HALLIS, Bassam
 SILMAN, Nigel
 SHONE, Clifford Charles
 SUTTON, John Mark

10 <120> Delivery of Superoxide Dismutase to Neuronal Cells

15 <130> 20994-SOD-heavy chain conjugates

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20 <150> GB 9824282.9
 <151> 1998-11-05

20 <160> 11

25 <170> PatentIn Ver. 2.1

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 35 40 45

40 Leu Gln Asn Lys Ser Leu Glu Leu Leu Ser Asn Leu Glu Ala Leu
 50 55 60

45 Pro Glu Ser Ile Arg Thr Ala Val Arg Asn Asn Gly Gly Gly His Ala
 65 70 75 80

45 Asn His Ser Leu Phe Trp Thr Ile Leu Ser Pro Asn Gly Gly Glu
 85 90 95

50 Pro Thr Gly Glu Leu Ala Glu Ala Ile Asn Lys Lys Phe Gly Ser Phe
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50 Thr Ala Phe Lys Asp Glu Phe Ser Lys Ala Ala Gly Arg Phe Gly
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55 Ser Gly Trp Ala Trp Leu Val Val Asn Asn Gly Glu Leu Glu Ile Thr
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- 2 -

- 3 -

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Glu Val Ala Lys Arg Tyr Ser Glu Ala Lys Ala Lys Gln Arg Ser Cys
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30 Gly Leu Val Pro Arg Gly Ser Gly Pro Gly Ser Ala Leu Asn Asp Leu
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35 Cys Ile Lys Val Asn Asn Trp Asp Leu Phe Phe Ser Pro Ser Glu Asp
 225 230 235 240

Asn Phe Thr Asn Asp Leu Asn Lys Gly Glu Glu Ile Thr Ser Asp Thr
 245 250 255

40 Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Leu Asp Leu Ile Gln Gln
 260 265 270

Tyr Tyr Leu Thr Phe Asn Phe Asp Asn Glu Pro Glu Asn Ile Ser Ile
 275 280 285

45 Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu Glu Leu Met Pro Asn
 290 295 300

Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu Leu Asp Lys Tyr Thr
 305 310 315 320

Met Phe His Tyr Leu Arg Ala Gln Glu Phe Glu His Gly Lys Ser Arg
 325 330 335

55 Ile Ala Leu Thr Asn Ser Val Asn Glu Ala Leu Leu Asn Pro Ser Arg
 340 345 350

Val Tyr Thr Phe Phe Ser Ser Asp Tyr Val Lys Lys Val Asn Lys Ala
 355 360 365

60 Thr Glu Ala Ala Met Phe Leu Gly Trp Val Glu Gln Leu Val Tyr Asp
 370 375 380

Phe Thr Asp Glu Thr Ser Glu Val Ser Thr Thr Asp Lys Ile Ala Asp
 385 390 395 400

Ile Thr Ile Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Gly Asn

- 4 -

	405	410	415
	Met Leu Tyr Lys Asp Asp Phe Val Gly Ala Leu Ile Phe Ser Gly Ala		
5	420	425	430
	Val Ile Leu Leu Glu Phe Ile Pro Glu Ile Ala Ile Pro Val Leu Gly		
	435	440	445
10	Thr Phe Ala Leu Val Ser Tyr Ile Ala Asn Lys Val Leu Thr Val Gln		
	450	455	460
	Thr Ile Asp Asn Ala Leu Ser Lys Arg Asn Glu Lys Trp Asp Glu Val		
	465	470	475
15	Tyr Lys Tyr Ile Val Thr Asn Trp Leu Ala Lys Val Asn Thr Gln Ile		
	485	490	495
	Asp Leu Ile Arg Lys Lys Met Lys Glu Ala Leu Glu Asn Gln Ala Glu		
20	500	505	510
	Ala Thr Lys Ala Ile Ile Asn Tyr Gln Tyr Asn Gln Tyr Thr Glu Glu		
	515	520	525
25	Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp Leu Ser Ser Lys Leu		
	530	535	540
	Asn Glu Ser Ile Asn Lys Ala Met Ile Asn Ile Asn Lys Phe Leu Asn		
	545	550	555
30	Gln Cys Ser Val Ser Tyr Leu Met Asn Ser Met Ile Pro Tyr Gly Val		
	565	570	575
	Lys Arg Leu Glu Asp Phe Asp Ala Ser Leu Lys Asp Ala Leu Leu Lys		
35	580	585	590
	Tyr Ile Tyr Asp Asn Arg Gly Thr Leu Ile Gly Gln Val Asp Arg Leu		
	595	600	605
40	Lys Asp Lys Val Asn Asn Thr Leu Ser Thr Asp Ile Pro Phe Gln Leu		
	610	615	620
	Ser Lys Tyr Val Asp Asn Gln Arg Leu Leu Ser Thr Phe Thr Glu Tyr		
	625	630	635
45	640		
	Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg Tyr Glu Ser		
	645	650	655
	Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile Asn Ile Gly		
50	660	665	670
	Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile Gln Leu Phe		
	675	680	685
55	Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn Ala Ile Val		
	690	695	700
	Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp Ile Arg Ile		
	705	710	715
60	720		
	Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr Thr Ile Ile		
	725	730	735
	Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu Asn Tyr Gly		
65	740	745	750
	Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys Gln Arg Val		
	755	760	765

- 5 -

Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr Ile Asn Arg
 770 775 780
 5 Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn Ser Lys Ile
 785 790 795 800
 Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser Asn Leu Gly
 805 810 815
 10 Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp Gly Cys Arg
 820 825 830
 Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu Phe Asp Lys
 835 840 845
 15 Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn Gln Ser Asn
 850 855 860
 20 Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln Tyr Asp Lys
 865 870 875 880
 Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr Val Asp Val
 885 890 895
 25 Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly Pro Arg Gly
 900 905 910
 Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu Tyr Arg Gly
 915 920 925
 30 Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys Asp Asn Ile
 930 935 940
 35 Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val Lys Asn Lys
 945 950 955 960
 Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val Glu Lys Ile
 965 970 975
 40 Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser Gln Val Val
 980 985 990
 Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys Cys Lys Met
 995 1000 1005
 45 Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile Gly Phe His
 1010 1015 1020
 50 Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp Tyr Asn Arg
 1025 1030 1035 1040
 Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp Glu Phe Ile
 1045 1050 1055
 55 Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu
 1060 1065
 60 <210> 4
 <211> 1070
 <212> PRT
 <213> Artificial Sequence
 65 <220>
 <223> Description of Artificial Sequence:construct

- 6 -

<400> 4
 Met Pro Phe Glu Leu Pro Ala Leu Pro Tyr Pro Tyr Asp Ala Leu Glu
 1 5 10 15

5 Pro His Ile Asp Lys Glu Thr Met Asn Ile His His Thr Lys His His
 20 25 30

Asn Thr Tyr Val Thr Asn Leu Asn Ala Ala Leu Glu Gly His Pro Asp
 10 35 40 45

Leu Gln Asn Lys Ser Leu Glu Glu Leu Leu Ser Asn Leu Glu Ala Leu
 50 55 60

15 Pro Glu Ser Ile Arg Thr Ala Val Arg Asn Asn Gly Gly Gly His Ala
 65 70 75 80

Asn His Ser Leu Phe Trp Thr Ile Leu Ser Pro Asn Gly Gly Glu
 20 85 90 95

Pro Thr Gly Glu Leu Ala Asp Ala Ile Asn Lys Lys Phe Gly Ser Phe
 100 105 110

Thr Ala Phe Lys Asp Glu Phe Ser Lys Ala Ala Ala Gly Arg Phe Gly
 25 115 120 125

Ser Gly Trp Ala Trp Leu Val Val Asn Asn Gly Glu Leu Glu Ile Thr
 130 135 140

Ser Thr Pro Asn Gln Asp Ser Pro Ile Met Glu Gly Lys Thr Pro Ile
 30 145 150 155 160

Leu Gly Leu Asp Val Trp Glu His Ala Tyr Tyr Leu Lys Tyr Gln Asn
 165 170 175

Arg Arg Pro Glu Tyr Ile Ala Ala Phe Trp Asn Val Val Asn Trp Asp
 35 180 185 190

Glu Val Ala Lys Arg Tyr Ser Glu Ala Lys Ala Lys Gln Arg Ser Cys
 40 195 200 205

Gly Leu Val Pro Arg Gly Ser Gly Pro Gly Ser Lys Ala Pro Gly Ile
 210 215 220

Cys Ile Asp Val Asp Asn Glu Asp Leu Phe Phe Ile Ala Asp Lys Asn
 45 225 230 235 240

Ser Phe Ser Asp Asp Leu Ser Lys Asn Glu Arg Ile Glu Tyr Asn Thr
 245 250 255

Gln Ser Asn Tyr Ile Glu Asn Asp Phe Pro Ile Asn Glu Leu Ile Leu
 50 260 265 270

Asp Thr Asp Leu Ile Ser Lys Ile Glu Leu Pro Ser Glu Asn Thr Glu
 55 275 280 285

Ser Leu Thr Asp Phe Asn Val Asp Val Pro Val Tyr Glu Lys Gln Pro
 290 295 300

Ala Ile Lys Lys Ile Phe Thr Asp Glu Asn Thr Ile Phe Gln Tyr Leu
 60 305 310 315 320

Tyr Ser Gln Thr Phe Pro Leu Asp Ile Arg Asp Ile Ser Leu Thr Ser
 325 330 335

Ser Phe Asp Asp Ala Leu Leu Phe Ser Asn Lys Val Tyr Ser Phe Phe
 65 340 345 350

- 7 -

Ser Met Asp Tyr Ile Lys Thr Ala Asn Lys Val Val Glu Ala Gly Leu
 355 360 365
 5 Phe Ala Gly Trp Val Lys Gln Ile Val Asn Asp Phe Val Ile Glu Ala
 370 375 380
 Asn Lys Ser Asn Thr Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val
 385 390 395 400
 10 Pro Tyr Ile Gly Leu Ala Leu Asn Val Gly Asn Glu Thr Ala Lys Gly
 405 410 415
 Asn Phe Glu Asn Ala Phe Glu Ile Ala Gly Ala Ser Ile Leu Leu Glu
 420 425 430
 15 Phe Ile Pro Glu Leu Leu Ile Pro Val Val Gly Ala Phe Leu Leu Glu
 435 440 445
 20 Ser Tyr Ile Asp Asn Lys Asn Lys Ile Ile Lys Thr Ile Asp Asn Ala
 450 455 460
 Leu Thr Lys Arg Asn Glu Lys Trp Ser Asp Met Tyr Gly Leu Ile Val
 465 470 475 480
 25 Ala Gln Trp Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile Lys Glu
 485 490 495
 Gly Met Tyr Lys Ala Leu Asn Tyr Gln Ala Gln Ala Leu Glu Ile
 500 505 510
 30 Ile Lys Tyr Arg Tyr Asn Ile Tyr Ser Glu Lys Glu Lys Ser Asn Ile
 515 520 525
 35 Asn Ile Asp Phe Asn Asp Ile Asn Ser Lys Leu Asn Glu Gly Ile Asn
 530 535 540
 Gln Ala Ile Asp Asn Ile Asn Asn Phe Ile Asn Gly Cys Ser Val Ser
 545 550 555 560
 40 Tyr Leu Met Lys Lys Met Ile Pro Leu Ala Val Glu Lys Leu Leu Asp
 565 570 575
 Phe Asp Asn Thr Leu Lys Lys Asn Leu Leu Asn Tyr Ile Asp Glu Asn
 580 585 590
 45 Lys Leu Tyr Leu Ile Gly Ser Ala Glu Tyr Glu Lys Ser Lys Val Asn
 595 600 605
 50 Lys Tyr Leu Lys Thr Ile Met Pro Phe Asp Leu Ser Ile Tyr Thr Asn
 610 615 620
 Asp Thr Ile Leu Ile Glu Met Phe Asn Lys Tyr Asn Ser Glu Ile Leu
 625 630 635 640
 55 Asn Asn Ile Ile Leu Asn Leu Arg Tyr Lys Asp Asn Asn Leu Ile Asp
 645 650 655
 Leu Ser Gly Tyr Gly Ala Lys Val Glu Val Tyr Asp Gly Val Glu Leu
 660 665 670
 60 Asn Asp Lys Asn Gln Phe Lys Leu Thr Ser Ser Ala Asn Ser Lys Ile
 675 680 685
 Arg Val Thr Gln Asn Gln Asn Ile Ile Phe Asn Ser Val Phe Leu Asp
 690 695 700
 65 Phe Ser Val Ser Phe Trp Ile Arg Ile Pro Lys Tyr Lys Asn Asp Gly

- 8 -

	705	710	715	720
	Ile Gln Asn Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys			
	725		730	735
5	Asn Asn Ser Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp			
	740		745	750
10	Thr Leu Ile Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr			
	755		760	765
	Asn Ile Arg Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val			
	770		775	780
15	Thr Ile Thr Asn Asn Leu Asn Asn Ala Lys Ile Tyr Ile Asn Gly Lys			
	785		790	800
	Leu Glu Ser Asn Thr Asp Ile Lys Asp Ile Arg Glu Val Ile Ala Asn			
	805		810	815
20	Gly Glu Ile Ile Phe Lys Leu Asp Gly Asp Ile Asp Arg Thr Gln Phe			
	820		825	830
	Ile Trp Met Lys Tyr Phe Ser Ile Phe Asn Thr Glu Leu Ser Gln Ser			
25	835		840	845
	Asn Ile Glu Glu Arg Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys			
	850		855	860
30	Asp Phe Trp Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe			
	865		870	880
	Asn Ala Gly Asn Lys Asn Ser Tyr Ile Lys Leu Lys Lys Asp Ser Pro			
	885		890	895
35	Val Gly Glu Ile Leu Thr Arg Ser Lys Tyr Asn Gln Asn Ser Lys Tyr			
	900		905	910
40	Ile Asn Tyr Arg Asp Leu Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg			
	915		920	925
	Lys Ser Asn Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp			
	930		935	940
45	Tyr Ile Tyr Leu Asp Phe Phe Asn Leu Asn Gln Glu Trp Arg Val Tyr			
	945		950	960
	Thr Tyr Lys Tyr Phe Lys Glu Glu Lys Leu Phe Leu Ala Pro			
	965		970	975
50	Ile Ser Asp Ser Asp Glu Phe Tyr Asn Thr Ile Gln Ile Lys Glu Tyr			
	980		985	990
	Asp Glu Gln Pro Thr Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu			
55	995		1000	1005
	Glu Ser Thr Asp Glu Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu			
	1010		1015	1020
60	Ser Gly Ile Val Phe Glu Glu Tyr Lys Asp Tyr Phe Cys Ile Ser Lys			
	1025		1030	1040
	Trp Tyr Leu Lys Glu Val Lys Arg Lys Pro Tyr Asn Leu Lys Leu Gly			
	1045		1050	1055
65	Cys Asn Trp Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu			
	1060		1065	1070

- 9 -

5 <210> 5
 <211> 1059
 <212> PRT
 <213> Artificial Sequence

10 <220>
 <223> Description of Artificial Sequence:construct

15 <400> 5
 Met Pro Phe Glu Leu Pro Ala Leu Pro Tyr Pro Tyr Asp Ala Leu Glu
 1 5 10 15

20 Pro His Ile Asp Lys Glu Thr Met Asn Ile His His Thr Lys His His
 20 25 30

25 Asn Thr Tyr Val Thr Asn Leu Asn Ala Ala Leu Glu Gly His Pro Asp
 35 35 40 45

30 Leu Gln Asn Lys Ser Leu Glu Glu Leu Leu Ser Asn Leu Glu Ala Leu
 50 55 60

35 Pro Glu Ser Ile Arg Thr Ala Val Arg Asn Asn Gly Gly Gly His Ala
 65 70 75 80

40 Asn His Ser Leu Phe Trp Thr Ile Leu Ser Pro Asn Gly Gly Glu
 85 85 90 95

45 Pro Thr Gly Glu Leu Ala Asp Ala Ile Asn Lys Lys Phe Gly Ser Phe
 100 105 110

50 Thr Ala Phe Lys Asp Glu Phe Ser Lys Ala Ala Ala Gly Arg Phe Gly
 115 120 125

55 Ser Gly Trp Ala Trp Leu Val Val Asn Asn Gly Glu Leu Glu Ile Thr
 130 135 140

60 Ser Thr Pro Asn Gln Asp Ser Pro Ile Met Glu Gly Lys Thr Pro Ile
 145 150 155 160

65 Leu Gly Leu Asp Val Trp Glu His Ala Tyr Tyr Leu Lys Tyr Gln Asn
 165 170 175

70 Arg Arg Pro Glu Tyr Ile Ala Ala Phe Trp Asn Val Val Asn Trp Asp
 180 185 190

75 Glu Val Ala Lys Arg Tyr Ser Glu Ala Lys Ala Lys Gln Arg Ser Cys
 195 200 205

80 Gly Leu Val Pro Arg Gly Ser Gly Pro Gly Ser Lys Ala Pro Pro Arg
 210 215 220

85 Leu Cys Ile Arg Val Asn Asn Arg Glu Leu Phe Phe Val Ala Ser Glu
 225 230 235 240

90 Ser Ser Tyr Asn Glu Asn Asp Ile Asn Thr Pro Lys Glu Ile Asp Asp
 245 250 255

95 Thr Thr Asn Leu Asn Asn Tyr Arg Asn Asn Leu Asp Glu Val Ile
 260 265 270

100 Leu Asp Tyr Asn Ser Glu Thr Ile Pro Gln Ile Ser Asn Gln Thr Leu
 275 280 285

105 Asn Thr Leu Val Gln Asp Asp Ser Tyr Val Pro Arg Tyr Asp Ser Asn
 290 295 300

- 10 -

Gly Thr Ser Glu Ile Glu Glu His Asn Val Val Asp Leu Asn Val Phe
 305 310 315 320

5 Phe Tyr Leu His Ala Gln Lys Val Pro Glu Gly Glu Thr Asn Ile Ser
 325 330 335

Leu Thr Ser Ser Ile Asp Thr Ala Leu Ser Glu Glu Ser Gln Val Tyr
 340 345 350

10 Thr Phe Phe Ser Ser Glu Phe Ile Asn Thr Ile Asn Lys Pro Val His
 355 360 365

Ala Ala Leu Phe Ile Ser Trp Ile Asn Gln Val Ile Arg Asp Phe Thr
 370 375 380

15 Thr Glu Ala Thr Gln Lys Ser Thr Phe Asp Lys Ile Ala Asp Ile Ser
 385 390 395 400

20 Leu Val Val Pro Tyr Val Gly Leu Ala Leu Asn Ile Gly Asn Glu Val
 405 410 415

Gln Lys Glu Asn Phe Lys Glu Ala Phe Glu Leu Leu Gly Ala Gly Ile
 420 425 430

25 Leu Leu Glu Phe Val Pro Glu Leu Leu Ile Pro Thr Ile Leu Val Phe
 435 440 445

Thr Ile Lys Ser Phe Ile Gly Ser Ser Glu Asn Lys Asn Lys Ile Ile
 450 455 460

30 Lys Ala Ile Asn Asn Ser Leu Met Glu Arg Glu Thr Lys Trp Lys Glu
 465 470 475 480

35 Ile Tyr Ser Trp Ile Val Ser Asn Trp Leu Thr Arg Ile Asn Thr Gln
 485 490 495

Phe Asn Lys Arg Lys Glu Gln Met Tyr Gln Ala Leu Gln Asn Gln Val
 500 505 510

40 Asp Ala Ile Lys Thr Val Ile Glu Tyr Lys Tyr Asn Asn Tyr Thr Ser
 515 520 525

Asp Glu Arg Asn Arg Leu Glu Ser Glu Tyr Asn Ile Asn Asn Ile Arg
 530 535 540

45 Glu Glu Leu Asn Lys Lys Val Ser Leu Ala Met Glu Asn Ile Glu Arg
 545 550 555 560

50 Phe Ile Thr Glu Ser Ser Ile Phe Tyr Leu Met Lys Leu Ile Asn Glu
 565 570 575

Ala Lys Val Ser Lys Leu Arg Glu Tyr Asp Glu Gly Val Lys Glu Tyr
 580 585 590

55 Leu Leu Asp Tyr Ile Ser Glu His Arg Ser Ile Leu Gly Asn Ser Val
 595 600 605

Gln Glu Leu Asn Asp Leu Val Thr Ser Thr Leu Asn Asn Ser Ile Pro
 610 615 620

60 Phe Glu Leu Ser Ser Tyr Thr Asn Asp Lys Ile Leu Ile Leu Tyr Phe
 625 630 635 640

65 Asn Lys Leu Tyr Lys Lys Ile Lys Asp Asn Ser Ile Leu Asp Met Arg
 645 650 655

Tyr Glu Asn Asn Lys Phe Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile

- 11 -

	660	665	670	
	Ser Ile Asn Gly Asp Val Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe			
	675	680	685	
5	Gly Ile Tyr Ser Ser Lys Pro Ser Glu Val Asn Ile Ala Gln Asn Asn			
	690	695	700	
10	Asp Ile Ile Tyr Asn Gly Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp			
	705	710	715	720
	Val Arg Ile Pro Lys Tyr Phe Asn Lys Val Asn Leu Asn Asn Glu Tyr			
	725	730	735	
15	Thr Ile Ile Asp Cys Ile Arg Asn Asn Asn Ser Gly Trp Lys Ile Ser			
	740	745	750	
	Leu Asn Tyr Asn Lys Ile Ile Trp Thr Leu Gln Asp Thr Ala Gly Asn			
	755	760	765	
20	Asn Gln Lys Leu Val Phe Asn Tyr Thr Gln Met Ile Ser Ile Ser Asp			
	770	775	780	
25	Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Gly			
	785	790	795	800
	Asn Ser Arg Ile Tyr Ile Asn Gly Asn Leu Ile Asp Glu Lys Ser Ile			
	805	810	815	
30	Ser Asn Leu Gly Asp Ile His Val Ser Asp Asn Ile Leu Phe Lys Ile			
	820	825	830	
	Val Gly Cys Asn Asp Thr Arg Tyr Val Gly Ile Arg Tyr Phe Lys Val			
	835	840	845	
35	Phe Asp Thr Glu Leu Gly Lys Thr Glu Ile Glu Thr Leu Tyr Ser Asp			
	850	855	860	
40	Glu Pro Asp Pro Ser Ile Leu Lys Asp Phe Trp Gly Asn Tyr Leu Leu			
	865	870	875	880
	Tyr Asn Lys Arg Tyr Tyr Leu Leu Asn Leu Leu Arg Thr Asp Lys Ser			
	885	890	895	
45	Ile Thr Gln Asn Ser Asn Phe Leu Asn Ile Asn Gln Gln Arg Gly Val			
	900	905	910	
	Tyr Gln Lys Pro Asn Ile Phe Ser Asn Thr Arg Leu Tyr Thr Gly Val			
	915	920	925	
50	Glu Val Ile Ile Arg Lys Asn Gly Ser Thr Asp Ile Ser Asn Thr Asp			
	930	935	940	
55	Asn Phe Val Arg Lys Asn Asp Leu Ala Tyr Ile Asn Val Val Asp Arg			
	945	950	955	960
	Asp Val Glu Tyr Arg Leu Tyr Ala Asp Ile Ser Ile Ala Lys Pro Glu			
	965	970	975	
60	Lys Ile Ile Lys Leu Ile Arg Thr Ser Asn Ser Asn Asn Ser Leu Gly			
	980	985	990	
	Gln Ile Ile Val Met Asp Ser Ile Gly Asn Asn Cys Thr Met Asn Phe			
	995	1000	1005	
65	Gln Asn Asn Asn Gly Gly Asn Ile Gly Leu Leu Gly Phe His Ser Asn			
	1010	1015	1020	

- 12 -

Asn Leu Val Ala Ser Ser Trp Tyr Tyr Asn Asn Ile Arg Lys Asn Thr
1025 1030 1035 1040

Ser Ser Asn Gly Cys Phe Trp Ser Phe Ile Ser Lys Glu His Gly Trp
5 1045 1050 1055

Gln Glu Asn

10

<210> 6
<211> 1092
<212> PRT
15 <213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:construct

20 <400> 6
Met Leu Ser Arg Ala Val Cys Gly Thr Ser Arg Gln Leu Ala Pro Ala
1 5 10 15

Leu Gly Tyr Leu Gly Ser Arg Gln Lys His Ser Arg Gly Ser Pro Ala
25 20 25 30

Leu Pro Tyr Pro Tyr Asp Ala Leu Glu Pro His Ile Asp Lys Glu Thr
35 40 45

30 Met Asn Ile His His Thr Lys His His Asn Thr Tyr Val Thr Asn Leu
50 55 60

Asn Ala Ala Leu Glu Gly His Pro Asp Leu Gln Asn Lys Ser Leu Glu
35 65 70 75 80

35 Glu Leu Leu Ser Asn Leu Glu Ala Leu Pro Glu Ser Ile Arg Thr Ala
85 90 95

40 Val Arg Asn Asn Gly Gly His Ala Asn His Ser Leu Phe Trp Thr
100 105 110

Ile Leu Ser Pro Asn Gly Gly Glu Pro Thr Gly Glu Leu Ala Asp
115 120 125

45 Ala Ile Asn Lys Lys Phe Gly Ser Phe Thr Ala Phe Lys Asp Glu Phe
130 135 140

Ser Lys Ala Ala Ala Gly Arg Phe Gly Ser Gly Trp Ala Trp Leu Val
45 145 150 155 160

50 Val Asn Asn Gly Glu Leu Glu Ile Thr Ser Thr Pro Asn Gln Asp Ser
165 170 175

55 Pro Ile Met Glu Gly Lys Thr Pro Ile Leu Gly Leu Asp Val Trp Glu
180 185 190

His Ala Tyr Tyr Leu Lys Tyr Gln Asn Arg Arg Pro Glu Tyr Ile Ala
195 200 205

60 Ala Phe Trp Asn Val Val Asn Trp Asp Glu Val Ala Lys Arg Tyr Ser
210 215 220

Glu Ala Lys Ala Lys Gln Arg Ser Cys Gly Leu Val Pro Arg Gly Ser
65 225 230 235 240

Gly Pro Gly Ser Ala Leu Asn Asp Leu Cys Ile Lys Val Asn Asn Trp
245 250 255

- 13 -

Asp Leu Phe Phe Ser Pro Ser Glu Asp Asn Phe Thr Asn Asp Leu Asn
 260 265 270
 5 Lys Gly Glu Glu Ile Thr Ser Asp Thr Asn Ile Glu Ala Ala Glu Glu
 275 280 285
 Asn Ile Ser Leu Asp Leu Ile Gln Gln Tyr Tyr Leu Thr Phe Asn Phe
 290 295 300
 10 Asp Asn Glu Pro Glu Asn Ile Ser Ile Glu Asn Leu Ser Ser Asp Ile
 305 310 315 320
 Ile Gly Gln Leu Glu Leu Met Pro Asn Ile Glu Arg Phe Pro Asn Gly
 325 330 335
 15 Lys Lys Tyr Glu Leu Asp Lys Tyr Thr Met Phe His Tyr Leu Arg Ala
 340 345 350
 Gln Glu Phe Glu His Gly Lys Ser Arg Ile Ala Leu Thr Asn Ser Val
 20 355 360 365
 Asn Glu Ala Leu Leu Asn Pro Ser Arg Val Tyr Thr Phe Phe Ser Ser
 370 375 380
 25 Asp Tyr Val Lys Lys Val Asn Lys Ala Thr Glu Ala Ala Met Phe Leu
 385 390 395 400
 Gly Trp Val Glu Gln Leu Val Tyr Asp Phe Thr Asp Glu Thr Ser Glu
 405 410 415
 30 Val Ser Thr Thr Asp Lys Ile Ala Asp Ile Thr Ile Ile Pro Tyr
 420 425 430
 Ile Gly Pro Ala Leu Asn Ile Gly Asn Met Leu Tyr Lys Asp Asp Phe
 35 435 440 445
 Val Gly Ala Leu Ile Phe Ser Gly Ala Val Ile Leu Leu Glu Phe Ile
 450 455 460
 40 Pro Glu Ile Ala Ile Pro Val Leu Gly Thr Phe Ala Leu Val Ser Tyr
 465 470 475 480
 Ile Ala Asn Lys Val Leu Thr Val Gln Thr Ile Asp Asn Ala Leu Ser
 485 490 495
 45 Lys Arg Asn Glu Lys Trp Asp Glu Val Tyr Lys Tyr Ile Val Thr Asn
 500 505 510
 Trp Leu Ala Lys Val Asn Thr Gln Ile Asp Leu Ile Arg Lys Lys Met
 515 520 525
 Lys Glu Ala Leu Glu Asn Gln Ala Glu Ala Thr Lys Ala Ile Ile Asn
 530 535 540
 55 Tyr Gln Tyr Asn Gln Tyr Thr Glu Glu Glu Lys Asn Asn Ile Asn Phe
 545 550 555 560
 Asn Ile Asp Asp Leu Ser Ser Lys Leu Asn Glu Ser Ile Asn Lys Ala
 565 570 575
 60 Met Ile Asn Ile Asn Lys Phe Leu Asn Gln Cys Ser Val Ser Tyr Leu
 580 585 590
 Met Asn Ser Met Ile Pro Tyr Gly Val Lys Arg Leu Glu Asp Phe Asp
 65 595 600 605
 Ala Ser Leu Lys Asp Ala Leu Leu Lys Tyr Ile Tyr Asn Arg Gly

- 14 -

	610	615	620													
	Thr	Leu	Ile	Gly	Gln	Val	Asp	Arg	Leu	Lys	Asp	Lys	Val	Asn	Asn	Thr
5	625				630				635				640			
	Leu	Ser	Thr	Asp	Ile	Pro	Phe	Gln	Leu	Ser	Lys	Tyr	Val	Asp	Asn	Gln
		645						650			655					
10	Arg	Leu	Leu	Ser	Thr	Phe	Thr	Glu	Ile	Lys	Asn	Ile	Ile	Asn	Thr	
		660						665			670					
	Ser	Ile	Leu	Asn	Leu	Arg	Tyr	Glu	Ser	Asn	His	Leu	Ile	Asp	Leu	Ser
		675						680			685					
15	Arg	Tyr	Ala	Ser	Lys	Ile	Asn	Ile	Gly	Ser	Lys	Val	Asn	Phe	Asp	Pro
		690					695			700						
	Ile	Asp	Lys	Asn	Gln	Ile	Gln	Leu	Phe	Asn	Leu	Glu	Ser	Ser	Lys	Ile
		705					710			715			720			
20	Glu	Val	Ile	Leu	Lys	Asn	Ala	Ile	Val	Tyr	Asn	Ser	Met	Tyr	Glu	Asn
		725					730			735						
25	Phe	Ser	Thr	Ser	Phe	Trp	Ile	Arg	Ile	Pro	Lys	Tyr	Phe	Asn	Ser	Ile
		740					745			750						
	Ser	Leu	Asn	Asn	Glu	Tyr	Thr	Ile	Ile	Asn	Cys	Met	Glu	Asn	Asn	Ser
		755					760			765						
30	Gly	Trp	Lys	Val	Ser	Leu	Asn	Tyr	Gly	Glu	Ile	Ile	Trp	Thr	Leu	Gln
		770					775			780						
	Asp	Thr	Gln	Glu	Ile	Lys	Gln	Arg	Val	Val	Phe	Lys	Tyr	Ser	Gln	Met
		785				790			795			800				
35	Ile	Asn	Ile	Ser	Asp	Tyr	Ile	Asn	Arg	Trp	Ile	Phe	Val	Thr	Ile	Thr
		805					810			815						
40	Asn	Asn	Arg	Leu	Asn	Asn	Ser	Lys	Ile	Tyr	Ile	Asn	Gly	Arg	Leu	Ile
		820					825			830						
	Asp	Gln	Lys	Pro	Ile	Ser	Asn	Leu	Gly	Asn	Ile	His	Ala	Ser	Asn	Asn
		835					840			845						
45	Ile	Met	Phe	Lys	Leu	Asp	Gly	Cys	Arg	Asp	Thr	His	Arg	Tyr	Ile	Trp
		850					855			860						
	Ile	Lys	Tyr	Phe	Asn	Leu	Phe	Asp	Lys	Glu	Leu	Asn	Glu	Lys	Glu	Ile
		865					870			875			880			
50	Lys	Asp	Leu	Tyr	Asp	Asn	Gln	Ser	Asn	Ser	Gly	Ile	Leu	Lys	Asp	Phe
							885			890			895			
	Trp	Gly	Asp	Tyr	Leu	Gln	Tyr	Asp	Lys	Pro	Tyr	Tyr	Met	Leu	Asn	Leu
							900			905			910			
55	Tyr	Asp	Pro	Asn	Lys	Tyr	Val	Asp	Val	Asn	Asn	Val	Gly	Ile	Arg	Gly
							915			920			925			
60	Tyr	Met	Tyr	Leu	Lys	Gly	Pro	Arg	Gly	Ser	Val	Met	Thr	Thr	Asn	Ile
							930			935			940			
	Tyr	Leu	Asn	Ser	Ser	Leu	Tyr	Arg	Gly	Thr	Lys	Phe	Ile	Ile	Lys	Lys
		945					950			955			960			
65	Tyr	Ala	Ser	Gly	Asn	Lys	Asp	Asn	Ile	Val	Arg	Asn	Asn	Asp	Arg	Val
									965			970			975	

- 15 -

Tyr Ile Asn Val Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn
 980 985 990
 5 Ala Ser Gln Ala Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro
 995 1000 1005
 Asp Val Gly Asn Leu Ser Gln Val Val Val Met Lys Ser Lys Asn Asp
 1010 1015 1020
 10 Gln Gly Ile Thr Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly
 1025 1030 1035 1040
 Asn Asp Ile Gly Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys
 1045 1050 1055
 15 Leu Val Ala Ser Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg
 1060 1065 1070
 20 Thr Leu Gly Cys Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly
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 Glu Arg Pro Leu
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 20 25 30
 Leu Pro Tyr Pro Tyr Asp Ala Leu Glu Pro His Ile Asp Lys Glu Thr
 35 40 45
 45 Met Asn Ile His His Thr Lys His His Asn Thr Tyr Val Thr Asn Leu
 50 55 60
 Asn Ala Ala Leu Glu Gly His Pro Asp Leu Gln Asn Lys Ser Leu Glu
 65 70 75 80
 50 Glu Leu Leu Ser Asn Leu Glu Ala Leu Pro Glu Ser Ile Arg Thr Ala
 85 90 95
 55 Val Arg Asn Asn Gly Gly His Ala Asn His Ser Leu Phe Trp Thr
 100 105 110
 Ile Leu Ser Pro Asn Gly Gly Glu Pro Thr Gly Glu Leu Ala Asp
 115 120 125
 60 Ala Ile Asn Lys Lys Phe Gly Ser Phe Thr Ala Phe Lys Asp Glu Phe
 130 135 140
 Ser Lys Ala Ala Ala Gly Arg Phe Gly Ser Gly Trp Ala Trp Leu Val
 145 150 155 160
 65 Val Asn Asn Gly Glu Leu Glu Ile Thr Ser Thr Pro Asn Gln Asp Ser
 165 170 175

- 16 -

Pro Ile Met Glu Gly Lys Thr Pro Ile Leu Gly Leu Asp Val Trp Glu
 180 185 190
 His Ala Tyr Tyr Leu Lys Tyr Gln Asn Arg Arg Pro Glu Tyr Ile Ala
 5 195 200 205
 Ala Phe Trp Asn Val Val Asn Trp Asp Glu Val Ala Lys Arg Tyr Ser
 210 215 220
 10 Glu Ala Lys Ala Lys Gln Arg Ser Cys Gly Leu Val Pro Arg Gly Ser
 225 230 235 240
 Gly Pro Gly Ser Lys Ala Pro Gly Ile Cys Ile Asp Val Asp Asn Glu
 245 250 255
 15 Asp Leu Phe Phe Ile Ala Asp Lys Asn Ser Phe Ser Asp Asp Leu Ser
 260 265 270
 20 Lys Asn Glu Arg Ile Glu Tyr Asn Thr Gln Ser Asn Tyr Ile Glu Asn
 275 280 285
 Asp Phe Pro Ile Asn Glu Leu Ile Leu Asp Thr Asp Leu Ile Ser Lys
 290 295 300
 25 Ile Glu Leu Pro Ser Glu Asn Thr Glu Ser Leu Thr Asp Phe Asn Val
 305 310 315 320
 Asp Val Pro Val Tyr Glu Lys Gln Pro Ala Ile Lys Lys Ile Phe Thr
 325 330 335
 30 Asp Glu Asn Thr Ile Phe Gln Tyr Leu Tyr Ser Gln Thr Phe Pro Leu
 340 345 350
 35 Asp Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp Asp Ala Leu Leu
 355 360 365
 Phe Ser Asn Lys Val Tyr Ser Phe Phe Ser Met Asp Tyr Ile Lys Thr
 370 375 380
 40 Ala Asn Lys Val Val Glu Ala Gly Leu Phe Ala Gly Trp Val Lys Gln
 385 390 395 400
 Ile Val Asn Asp Phe Val Ile Glu Ala Asn Lys Ser Asn Thr Met Asp
 405 410 415
 45 Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile Gly Leu Ala Leu
 420 425 430
 Asn Val Gly Asn Glu Thr Ala Lys Gly Asn Phe Glu Asn Ala Phe Glu
 50 435 440 445
 Ile Ala Gly Ala Ser Ile Leu Leu Glu Phe Ile Pro Glu Leu Leu Ile
 450 455 460
 55 Pro Val Val Gly Ala Phe Leu Leu Glu Ser Tyr Ile Asp Asn Lys Asn
 465 470 475 480
 Lys Ile Ile Lys Thr Ile Asp Asn Ala Leu Thr Lys Arg Asn Glu Lys
 60 485 490 495
 Trp Ser Asp Met Tyr Gly Leu Ile Val Ala Gln Trp Leu Ser Thr Val
 500 505 510
 Asn Thr Gln Phe Tyr Thr Ile Lys Glu Gly Met Tyr Lys Ala Leu Asn
 65 515 520 525
 Tyr Gln Ala Gln Ala Leu Glu Glu Ile Ile Lys Tyr Arg Tyr Asn Ile

- 17 -

	530	535	540	
	Tyr Ser Glu Lys Glu Lys Ser Asn Ile Asn Ile Asp Phe Asn Asp Ile			
5	545	550	555	560
	Asn Ser Lys Leu Asn Glu Gly Ile Asn Gln Ala Ile Asp Asn Ile Asn			
	565	570	575	
10	Asn Phe Ile Asn Gly Cys Ser Val Ser Tyr Leu Met Lys Lys Met Ile			
	580	585	590	
	Pro Leu Ala Val Glu Lys Leu Leu Asp Phe Asp Asn Thr Leu Lys Lys			
	595	600	605	
15	Asn Leu Leu Asn Tyr Ile Asp Glu Asn Lys Leu Tyr Leu Ile Gly Ser.			
	610	615	620	
	Ala Glu Tyr Glu Lys Ser Lys Val Asn Lys Tyr Leu Lys Thr Ile Met			
20	625	630	635	640
	Pro Phe Asp Leu Ser Ile Tyr Thr Asn Asp Thr Ile Leu Ile Glu Met			
	645	650	655	
25	Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile Ile Leu Asn Leu			
	660	665	670	
	Arg Tyr Lys Asp Asn Asn Leu Ile Asp Leu Ser Gly Tyr Gly Ala Lys			
	675	680	685	
30	Val Glu Val Tyr Asp Gly Val Glu Leu Asn Asp Lys Asn Gln Phe Lys			
	690	695	700	
	Leu Thr Ser Ser Ala Asn Ser Lys Ile Arg Val Thr Gln Asn Gln Asn			
35	705	710	715	720
	Ile Ile Phe Asn Ser Val Phe Leu Asp Phe Ser Val Ser Phe Trp Ile			
	725	730	735	
40	Arg Ile Pro Lys Tyr Lys Asn Asp Gly Ile Gln Asn Tyr Ile His Asn			
	740	745	750	
	Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser Gly Trp Lys Ile			
	755	760	765	
45	Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile Asp Ile Asn Gly			
	770	775	780	
	Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg Glu Asp Ile Ser			
50	785	790	795	800
	Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr Asn Asn Leu Asn			
	805	810	815	
55	Asn Ala Lys Ile Tyr Ile Asn Gly Lys Leu Glu Ser Asn Thr Asp Ile			
	820	825	830	
	Lys Asp Ile Arg Glu Val Ile Ala Asn Gly Glu Ile Ile Phe Lys Leu			
	835	840	845	
60	Asp Gly Asp Ile Asp Arg Thr Gln Phe Ile Trp Met Lys Tyr Phe Ser			
	850	855	860	
	Ile Phe Asn Thr Glu Leu Ser Gln Ser Asn Ile Glu Glu Arg Tyr Lys			
65	865	870	875	880
	Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp Gly Asn Pro Leu			
	885	890	895	

- 18 -

Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly Asn Lys Asn Ser
 900 905 910
 Tyr Ile Lys Leu Lys Lys Asp Ser Pro Val Gly Glu Ile Leu Thr Arg
 5 915 920 925
 Ser Lys Tyr Asn Gln Asn Ser Lys Tyr Ile Asn Tyr Arg Asp Leu Tyr
 930 935 940
 10 Ile Gly Glu Lys Phe Ile Ile Arg Arg Lys Ser Asn Ser Gln Ser Ile
 945 950 955 960
 Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr Leu Asp Phe Phe
 15 965 970 975
 Asn Leu Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys Tyr Phe Lys Lys
 980 985 990
 20 Glu Glu Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp Ser Asp Glu Phe
 995 1000 1005
 Tyr Asn Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln Pro Thr Tyr Ser
 1010 1015 1020
 25 Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr Asp Glu Ile Gly
 1025 1030 1035 1040
 Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Ile Val Phe Glu Glu
 30 1045 1050 1055
 Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu Lys Glu Val Lys
 1060 1065 1070
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 Lys Asp Glu Gly Trp Thr Glu
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 <211> 1084
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 45 <213> Artificial Sequence
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 Leu Gly Tyr Leu Gly Ser Arg Gln Lys His Ser Arg Gly Ser Pro Ala
 55 20 25 30
 Leu Pro Tyr Pro Tyr Asp Ala Leu Glu Pro His Ile Asp Lys Glu Thr
 35 40 45
 60 Met Asn Ile His His Thr Lys His His Asn Thr Tyr Val Thr Asn Leu
 50 55 60
 Asn Ala Ala Leu Glu Gly His Pro Asp Leu Gln Asn Lys Ser Leu Glu
 65 70 75 80
 65 Glu Leu Leu Ser Asn Leu Glu Ala Leu Pro Glu Ser Ile Arg Thr Ala
 85 90 95

- 19 -

Val Arg Asn Asn Gly Gly His Ala Asn His Ser Leu Phe Trp Thr
 100 105 110
 5 Ile Leu Ser Pro Asn Gly Gly Glu Pro Thr Gly Glu Leu Ala Asp
 115 120 125
 Ala Ile Asn Lys Lys Phe Gly Ser Phe Thr Ala Phe Lys Asp Glu Phe
 130 135 140
 10 Ser Lys Ala Ala Ala Gly Arg Phe Gly Ser Gly Trp Ala Trp Leu Val
 145 150 155 160
 Val Asn Asn Gly Glu Leu Glu Ile Thr Ser Thr Pro Asn Gln Asp Ser
 165 170 175
 15 Pro Ile Met Glu Gly Lys Thr Pro Ile Leu Gly Leu Asp Val Trp Glu
 180 185 190
 His Ala Tyr Tyr Leu Lys Tyr Gln Asn Arg Arg Pro Glu Tyr Ile Ala
 20 195 200 205
 Ala Phe Trp Asn Val Val Asn Trp Asp Glu Val Ala Lys Arg Tyr Ser
 210 215 220
 25 Glu Ala Lys Ala Lys Gln Arg Ser Cys Gly Leu Val Pro Arg Gly Ser
 225 230 235 240
 Gly Pro Gly Ser Lys Ala Pro Pro Arg Leu Cys Ile Arg Val Asn Asn
 245 250 255
 30 Arg Glu Leu Phe Phe Val Ala Ser Glu Ser Ser Tyr Asn Glu Asn Asp
 260 265 270
 Ile Asn Thr Pro Lys Glu Ile Asp Asp Thr Thr Asn Leu Asn Asn Asn
 35 275 280 285
 Tyr Arg Asn Asn Leu Asp Glu Val Ile Leu Asp Tyr Asn Ser Glu Thr
 290 295 300
 40 Ile Pro Gln Ile Ser Asn Gln Thr Leu Asn Thr Leu Val Gln Asp Asp
 305 310 315 320
 Ser Tyr Val Pro Arg Tyr Asp Ser Asn Gly Thr Ser Glu Ile Glu Glu
 325 330 335
 45 His Asn Val Val Asp Leu Asn Val Phe Phe Tyr Leu His Ala Gln Lys
 340 345 350
 Val Pro Glu Gly Glu Thr Asn Ile Ser Leu Thr Ser Ser Ile Asp Thr
 50 355 360 365
 Ala Leu Ser Glu Glu Ser Gln Val Tyr Thr Phe Phe Ser Ser Glu Phe
 370 375 380
 55 Ile Asn Thr Ile Asn Lys Pro Val His Ala Ala Leu Phe Ile Ser Trp
 385 390 395 400
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 60 405 410 415
 Thr Phe Asp Lys Ile Ala Asp Ile Ser Leu Val Val Pro Tyr Val Gly
 420 425 430
 65 Leu Ala Leu Asn Ile Gly Asn Glu Val Gln Lys Glu Asn Phe Lys Glu
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 Ala Phe Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Val Pro Glu

- 20 -

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	485 490 495		
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	500 505 510		
	Asn Trp Leu Thr Arg Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln		
	515 520 525		
15	Met Tyr Gln Ala Leu Gln Asn Gln Val Asp Ala Ile Lys Thr Val Ile		
	530 535 540		
	Glu Tyr Lys Tyr Asn Asn Tyr Thr Ser Asp Glu Arg Asn Arg Leu Glu		
20	545 550 555 560		
	Ser Glu Tyr Asn Ile Asn Asn Ile Arg Glu Glu Leu Asn Lys Lys Val		
	565 570 575		
25	Ser Leu Ala Met Glu Asn Ile Glu Arg Phe Ile Thr Glu Ser Ser Ile		
	580 585 590		
	Phe Tyr Leu Met Lys Leu Ile Asn Glu Ala Lys Val Ser Lys Leu Arg		
	595 600 605		
30	Glu Tyr Asp Glu Gly Val Lys Glu Tyr Leu Leu Asp Tyr Ile Ser Glu		
	610 615 620		
	His Arg Ser Ile Leu Gly Asn Ser Val Gln Glu Leu Asn Asp Leu Val		
35	625 630 635 640		
	Thr Ser Thr Leu Asn Asn Ser Ile Pro Phe Glu Leu Ser Ser Tyr Thr		
	645 650 655		
40	Asn Asp Lys Ile Leu Ile Leu Tyr Phe Asn Lys Leu Tyr Lys Lys Ile		
	660 665 670		
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	675 680 685		
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	690 695 700		
	Ile Tyr Ser Thr Asn Arg Asn Gln Phe Gly Ile Tyr Ser Ser Lys Pro		
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	755 760 765		
60	Asn Asn Asn Ser Gly Trp Lys Ile Ser Leu Asn Tyr Asn Lys Ile Ile		
	770 775 780		
	Trp Thr Leu Gln Asp Thr Ala Gly Asn Asn Gln Lys Leu Val Phe Asn		
65	785 790 795 800		
	Tyr Thr Gln Met Ile Ser Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe		
	805 810 815		

- 21 -

Val Thr Ile Thr Asn Asn Arg Leu Gly Asn Ser Arg Ile Tyr Ile Asn
 820 825 830
 Gly Asn Leu Ile Asp Glu Lys Ser Ile Ser Asn Leu Gly Asp Ile His
 5 835 840 845
 Val Ser Asp Asn Ile Leu Phe Lys Ile Val Gly Cys Asn Asp Thr Arg
 850 855 860
 10 Tyr Val Gly Ile Arg Tyr Phe Lys Val Phe Asp Thr Glu Leu Gly Lys
 865 870 875 880
 Thr Glu Ile Glu Thr Leu Tyr Ser Asp Glu Pro Asp Pro Ser Ile Leu
 885 890 895
 15 Lys Asp Phe Trp Gly Asn Tyr Leu Tyr Asn Lys Arg Tyr Tyr Leu
 900 905 910
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 20 915 920 925
 Leu Asn Ile Asn Gln Gln Arg Gly Val Tyr Gln Lys Pro Asn Ile Phe
 930 935 940
 25 Ser Asn Thr Arg Leu Tyr Thr Gly Val Glu Val Ile Ile Arg Lys Asn
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 Gly Ser Thr Asp Ile Ser Asn Thr Asp Asn Phe Val Arg Lys Asn Asp
 30 965 970 975
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 980 985 990
 35 Ala Asp Ile Ser Ile Ala Lys Pro Glu Lys Ile Ile Lys Leu Ile Arg
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 Thr Ser Asn Ser Asn Asn Ser Leu Gly Gln Ile Ile Val Met Asp Ser
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 Ile Gly Leu Leu Gly Phe His Ser Asn Asn Leu Val Ala Ser Ser Trp
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 55 <210> 9
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 comprising a mitochondrial leader from human MnSOD
 and B. Stearothermophilus SOD
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 1 5 10 15

- 22 -

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5 Leu Pro Tyr Pro Tyr Asp Ala Leu Glu Pro His Ile Asp Lys Glu Thr
35 40 45

Met Asn Ile His His Thr Lys His His Asn Thr Tyr Val Thr Asn Leu
50 55 60

10 Asn Ala Ala Leu Glu Gly His Pro Asp Leu Gln Asn Lys Ser Leu Glu
65 70 75 80

Glu Leu Leu Ser Asn Leu Glu Ala Leu Pro Glu Ser Ile Arg Thr Ala
85 90 95

15 Val Arg Asn Asn Gly Gly His Ala Asn His Ser Leu Phe Trp Thr
100 105 110

20 Ile Leu Ser Pro Asn Gly Gly Glu Pro Thr Gly Glu Leu Ala Asp
115 120 125

Ala Ile Asn Lys Lys Phe Gly Ser Phe Thr Ala Phe Lys Asp Glu Phe
130 135 140

25 Ser Lys Ala Ala Ala Gly Arg Phe Gly Ser Gly Trp Ala Trp Leu Val
145 150 155 160

Val Asn Asn Gly Glu Leu Glu Ile Thr Ser Thr Pro Asn Gln Asp Ser
165 170 175

30 Pro Ile Met Glu Gly Lys Thr Pro Ile Leu Gly Leu Asp Val Trp Glu
180 185 190

His Ala Tyr Tyr Leu Lys Tyr Gln Asn Arg Arg Pro Glu Tyr Ile Ala
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Ala Phe Trp Asn Val Val Asn Trp Asp Glu Val Ala Lys Arg Tyr Ser
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40 Glu Ala Lys Ala Lys
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45 <210> 10
<211> 24
<212> PRT
<213> Artificial Sequence

50 <220>
<223> Description of Artificial Sequence: modified human
mitochondrial leader sequence

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1 5 10 15

60 Leu Gly Tyr Leu Gly Ser Arg Gln
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65 <210> 11
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<212> PRT
<213> Artificial Sequence

- 23 -

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<223> Description of Artificial Sequence: modified human
mitochondrial leader sequence

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<400> 11
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1 5 10 15

10

Leu Gly Tyr Leu Gly Ser Arg Gln
20

INTERNATIONAL SEARCH REPORT

Intel. onal Application No
PCT/GB 99/03699

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/53 C12N15/62 C12N9/02 A61K38/44 A61K48/00
C07K14/33 A61K39/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FIGUEIREDO D M ET AL: "Delivery of recombinant tetanus -superoxide dismutase proteins to central nervous system neurons by retrograde axonal transport" EXPERIMENTAL NEUROLOGY, US, SAN DIEGO, CA, vol. 145, 1997, pages 546-554, XP002102526 the whole document	1,6,7,9, 10,13,15
Y	FUJII J ET AL: "A defect in the mitochondrial import of mutant Mn-superoxide dismutase produced in Sf21 cells." JOURNAL OF BIOCHEMISTRY, (1998 AUG) 124 (2) 340-6., XP000867725 page 340 -page 341; figures 1,5	16-22 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the International filing date
- "L" document which may throw doubt on priority, claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"a" document member of the same patent family

Date of the actual completion of the International search

Date of mailing of the International search report

28 February 2000

15/03/2000

Name and mailing address of the ISA
European Patent Office, P.B. 5618 Patentsteen 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Espen, J

1

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/03699

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOWLER C ET AL: "Characterization of the <i>Bacillus stearothermophilus</i> manganese superoxide dismutase gene and its ability to complement copper/zinc superoxide dismutase deficiency in <i>Saccharomyces cerevisiae</i> " JOURNAL OF BACTERIOLOGY, vol. 172, no. 3, 1990, pages 1539-1546, XP000877200 USA abstract	16-22
X	FRANCIS JW ET AL: "CuZn superoxide dismutase (SOD-1): tetanus toxin fragment C hybrid protein for targeted delivery of SOD-1 to neuronal cells" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 25, 1995, pages 15434-15442, XP002131795 MD US abstract; figures 1,4-8	1,6,7,9, 10,13,15
X	US 5 780 024 A (BROWN ROBERT H ET AL) 14 July 1998 (1998-07-14) the whole document	1,6,7,9, 10,13,15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/03699

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5780024	A 14-07-1998	NONE	